Društvo za Kliničku Genetiku Hrvatske
Clinical Genetic Society of Croatia

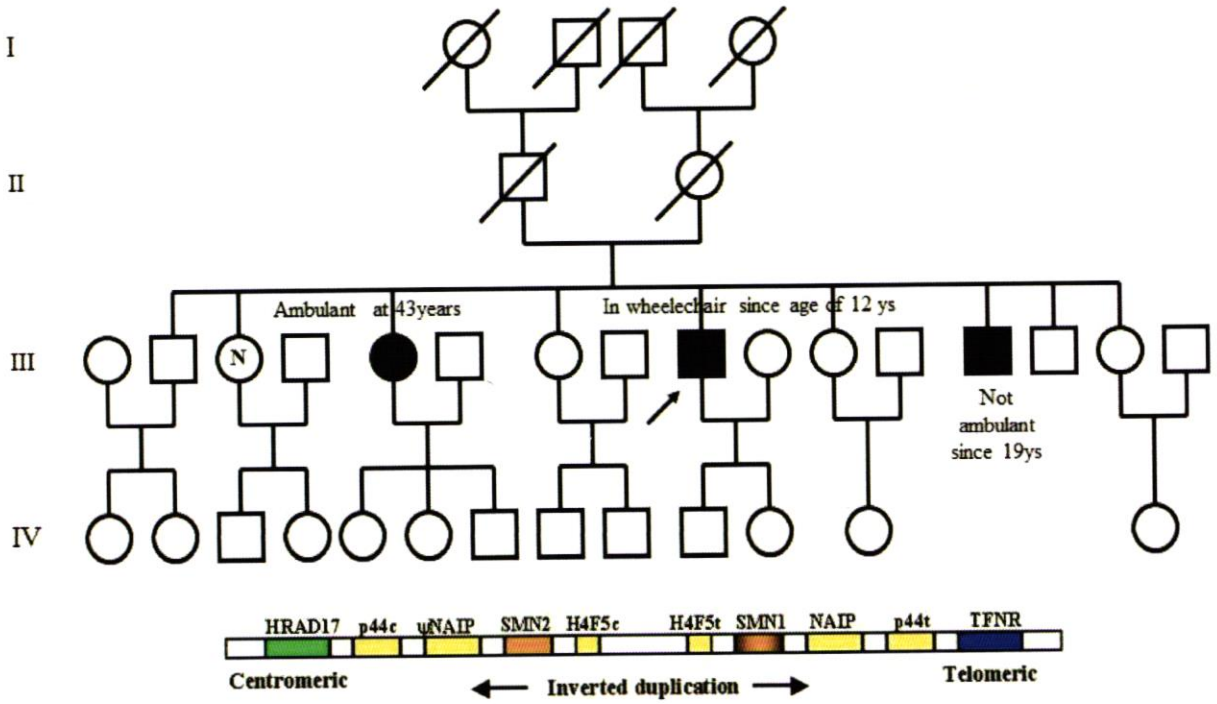
I

Medicinski fakultet Sveučilišta u Zagrebu
Zagreb University School of Medicine

DANI KLINIČKE GENETIKE: ŠTO JE NOVO?

Poslijediplomski tečaj stjecanja znanja s provjerom

DAYS OF CLINICAL GENETICS: WHAT'S NEW?



Hrvatski institut za istraživanje mozga, Zagreb
10. i 11. 6. 2016.
Croatian Institute for Brain Research, Zagreb, Croatia
June 10 & 11, 2016

Voditelj tečaja: Prof. dr. sc. Nina Canki-Klain, dr. med.
Director of the course: Prof. Nina Canki-Klain, MD, PhD

DANI KLINIČKE GENETIKE: ŠTO JE NOVO?
Poslijediplomski tečaj stjecanja znanja s provjerom
Postgraduate course of continuous education with testing
Zagreb, 10. i 11. 6. 2016.

Hrvatski institut za istraživanje mozga, Šalata 12
Croatian Institute for Brain Research

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PROGRAMME AND ABSTRACTS

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MEDICINSKA
NAKLADA

TABLE OF CONTENTS
Sadržaj

PROGRAMME
Program

DAYS OF CLINICAL GENETICS: WHAT'S NEW?

Friday, Juin 10, 2016

08:00-09:00	Registration and seting up of posters- Croatian Institut for Brain Research, Šalata 12,10 000 Zagreb, tel. 45 96 891
09:00-09:15	Opening Ceremony- Lecture Hall- Ground
09.15-11.00	Plenary Session I Chair: N.Canki-Klain, S.Levanat
09.15-10.00	30 years after the Duchenne muscular dystrophy gene discovery: What's new? Mireille Claustres Montpellier, France
10.00-10.45	Breast-ovarian cancer predispositions: which genes to test, why to test, who to test?" Dominique Stoppa Lyonnet Paris, France
10.45-11.00	Discussion
11.00-11.30	Coffee break
11.30-12.30	Oral presentations Chair: D. Lessel
11.30-11.50	Whole-exome Sequencing and Comparative Genomic Hybridization: complementary approaches in advanced genomics Oliver Vugrek Zagreb, Croatia
12.30-14.00	Lunch at Club of Croatian Institute for Brain Research

14.00-15.00	Poster Session Chair Lj.Šerman
15.00-16.45	Plenary Session II Chair: F.Bulić Jakuš, P.Korać
15.00-15.45	Genetics of neurocristopathy syndromes: a window on the non-coding genome Stanislas Lyonnet Paris, France
15.45-16.30	Genetics of progeroid syndromes Davor Lessel Hamburg, Germany Molecular genetics in breast cancer research Sonja Levanat Zagreb, Croatia
16.30-16.45	Discussion
16.45-17.00	Coffee break
17.00-18.30	Plenary Session III Chair: G.Šimić, N.Canki Klain
17.00-18.00	Spinal muscular atrophy (SMA): from gene and modifiers to therapy Brunhilde Wirth Cologne, Germany
18.00-18.30	Discussion on all topics of the day

DANI KLINIČKE GENETIKE:ŠTO JE NOVO?

Saturday, Juin 11, 2016

MULTIDISCIPLINARNI PRISTUP DIJAGNOSTICI I ZBRINJAVANJU OSOBA S NASLJEDNOM SKLONOŠĆU RAKU DOJKE I JAJNIKA

Predsjedatelji: Floriana Bulić-Jakuš i Stjepko Pleština

09:00	Budućnost onkologije u Hrvatskoj Stjepko Pleština
09.15	Multidisciplinarni pristup osobi s nasljednom sklonošću raku dojke i jajnika Nina Canki Klain
09:30	Genetika raka Nives Pećina-Šlaus
09:45	Genetska osnova raka dojke i jajnika Sonja Levanat
10:00	Deset godina testiranja BRCA1 i BRCA2 mutacija u Hrvatskoj Vesna Musani

10:15 Klinička dijagnostika i indikacije za genetsko testiranje raka dojke i jajnika
Natalija Dedić Plavetić

10:30 Genetsko savjetovanje prije i poslije testiranja
Tamara Žigman

10:45-11:15 RASPRAVA (predsjedatelji + svi predavači)

11:15-11:30 ODMOR

Predsjedatelji: Natalija Dedić Plavetić i Božena Šarčević

11:30 Uloga patologa u dijagnostici i terapiji raka dojke i jajnika
Božena Šarčević

11:45 Sadašnje mogućnosti i perspektive testiranja somatskih mutacija BRCA1,2 iz tkiva tumora jajnika
Blaženka Grahovac

12:00 Liječenje bolesnica s rakom dojke koje su nositeljice mutacija BRCA1 i BRCA2 gena
Paula Podolski

12:15 Praćenje bolesnica i zdravih nositeljica mutacija BRCA1 i BRCA2 gena
Ilona Sušac

12:30 Psihološki aspekti genetskog savjetovanja s osvrtom na procjenu rizika i informirani pristanak
Ljiljana Šerman

12:45 Informirani pristanak
Ana Borovečki

13:00-13:30 RASPRAVA

13:30-15:00 RUČAK u KLUBU HIIM-a

15:00 PISMENI TEST PROVJERE ZNANJA

15:45 EVALUACIJA TEČAJA I ZAKLJUČNA SJEDNICA

LECTURERS
Predavači

Prof. dr. sc. **Ana Borovečki**, dr. med., MF Zagreb, ŠNZ, Katedra za soc. med. i organizaciju zdravstvene zaštite

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Tamara Žigman, dr. med., KBC Sestre milosrdnice, Zagreb, Klinika za tumore, Genetsko savjetovalište

ABSTRACTS
SAŽECI

30 YEARS AFTER THE DUCHENNE MUSCULAR DYSTROPHY GENE DISCOVERY: WHAT'S NEW ?

Mireille CLAUSTRES

Founder and former head of the Molecular Genetic Laboratory for Rare Diseases & Inserm U827 at Montpellier's University Hospital, France.

Dystrophinopathies are X-linked recessive disorders caused by lack or dysfunction of dystrophin, a large scaffolding protein that normally confers mechanical stability to muscle fibres and ensures proper signalling across the sarcolemma. The most severe form, DMD, is characterized by progressive muscle deterioration with failed regeneration and replacement of muscle fibres with a fatty-fibrous matrix. Since the discovery of the DMD gene in 1987, continuous technical advances allowed to identify the genetic defects responsible for the different phenotypes and to understand their molecular consequences. Today, by combining genomic DNA techniques (MLPA, aCGH, Sanger sequencing, NGS) and mRNA analyses, the genetic cause can be detected in all patients with a dystrophinopathy. Nation-wide mutation databases are developed and display the distribution of large alterations (deletions, duplications, complex rearrangements) and point mutations typical of Duchenne or Becker phenotypes. Knowing precisely which DMD variant is causative of DMD or BMD is of major importance not only for diagnosis, genetic counselling, and prevention through prenatal or pre-implantation genetic diagnosis, but also for assessment of eligibility of DMD patients for clinical trials. The major modulator of phenotype severity is the DMD gene itself, through multiple alternative splicing events that change the composition of the mRNA or the structure of the encoded dystrophin and explain most of the exceptions to the Monaco's rule.

A multi-disciplinary clinical management approach is well recognised as a major factor in improved life expectancy of DMD patients (now 30 years), and there is an emphasis on utilising care guidelines to treat effectively the multiple clinical manifestations of DMD. Corticosteroids are so far the only medication proven to slow the progression of DMD, however they have limited efficacy and many adverse effects. During the past decade, there have been tremendous efforts towards gene and cell therapy to replace the defective DMD gene. Given their modest advances in clinical trials for DMD, other strategies based on the knowledge of the molecular defects have emerged. Ataluren is the first drug to target the underlying cause of DMD due to nonsense mutations, by promoting ribosomal readthrough of a PTC (premature termination codon) to produce full-length functional dystrophin. It is approved in Europe for the treatment of ambulatory patients ≥ 5 years old carrying a nonsense variant. Antisense oligonucleotide-based therapies modulate pre-mRNA splicing of the DMD gene to restore, through exon skipping, a viable reading frame and the expression of a shorter but semi-functional dystrophin that would mimick a "BMD" phenotype. Clinical trials using two different chemistries are conducted worldwide. A new promising genome-editing technology (CRISPR/Cas9*) can correct the gene through the cell's own mismatch repair mechanism. Spectacular results on mdx mouse and human DMD myoblasts have been published this year. Given that gene editing is a permanent reparation of the DMD gene, this treatment would not have to be repeated as it is the case for exon skipping induced by AONs.

Dystrophin replacement therapies to date have shown very limited ability to slow the disease and thus pharmacological strategies targeting other aspects of the pathological cascade are currently on the way. Many signalling pathways and cellular processes are altered downstream the missing dystrophin, including impaired calcium homeostasis, mitochondrial function, energy production, kinase activity, regeneration from stem cells, excessive production of reactive oxygene species, exposure to cytokines, inflammation, fibrosis... Dystrophin can be made only by muscle fibres, not by fibrotic or adipose tissue; as the necrotic/fibrotic/inflammatory processes start very early, there is need to develop in parallel dystrophin-independent therapies that improve overall muscle condition. It is likely that therapy of DMD will move towards combinatorial therapy. Researchers conducting DMD clinical trials face numerous challenges including the wide variability of disease progression between patients, the need for a standardized treatment approach and reliable outcome measures to monitor treatment efficacy in patients. The nearly 30 years of intensive research on dystrophinopathies not only led to a new understanding of the muscle cell membrane as well as new candidates for other forms of muscular dystrophy, but also benefited the field of human genetics.

**CRISPR/Cas9: Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR-associated (Cas) system.*

BREAST-OVARIAN CANCER PREDISPOSITIONS: WHICH GENES TO TEST, WHY TO TEST, WHO TO TEST?

Dominique Stoppa-Lyonnet
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CHAPTER 2
GENETICS OF OVARIAN CARCINOMAS

Claire Sénéchal[1*], Bruno Buecher[1], Antoine de Pauw[1], Claude Houdayer[1,2,3], Etienne Rouleau[1], Catherine Noguès[1,4], Dominique Stoppa-Lyonnet[1,2,3]

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ABSTRACT

It is estimated that 15 to 20% of ovarian carcinomas arise in women carrying a monoallelic germline cancer-predisposing gene mutation. The two main known hereditary forms of ovarian carcinoma are hereditary breast/ovarian cancer (HBOC) linked to BRCA1 or BRCA2 mutations, genes involved in DNA double-strand break repair by homologous recombination (HR) and Lynch syndrome linked to mutations in genes involved in DNA mismatch repair (MMR): MLH1, MSH2, MSH6 or PMS2. The contribution of BRCA1/2 mutations is at least tenfold higher than that of MMR genes. Identification of a cancer-predisposing mutation is useful for the prevention of breast, ovarian or colon cancers in affected women and their relatives and is now becoming a major part of the treatment of women with ovarian carcinoma, as Poly-ADP-ribose-polymerase (PARP) inhibitors have been demonstrated to be useful in HBOC syndrome. New perspectives are opening up in Lynch syndrome with immunotherapy targeting Lynch syndrome-related cancers, characterized by their immunogenicity. Other genes involved in the HR pathway (PALB2, RAD51, ATM ...) are good candidates to be associated with an increased risk of ovarian and breast cancers that would be expected to be also sensitive to PARP inhibitors. As the identification of women harbouring germline or tumour inactivation of HR genes and probably, in the near future, MMR gene mutations is now becoming essential for their treatment, increasing test demands and the need for rapid and complete analyses are going to modify current genetic counselling and testing practices .

Key-Words: Ovarian carcinoma, BRCA1, BRCA2, Lynch syndrome, HRness, patient information, epidemiological studies

2.1 Introduction

Up until now, the aim of genetic testing in a woman with ovarian carcinoma was to allow identification of a predisposing factor and therefore the prevention of other cancers as well as testing of her relatives in order to adapt their management. Ovarian cancer prevention of relatives has been and remains a major goal of cancer genetics, particularly because of the absence of reliable ovarian cancer screening. The scope of genetic testing has now been widened, especially in women carrying a germline BRCA1 or BRCA2 mutation: identification of such a mutation may change the treatment of the disease, and may therefore have a major impact on the patient's medical management.

Two main ovarian carcinoma (OC) genetic predisposion syndromes have been identified to date. Hereditary Breast and Ovarian Cancer (HBOC) syndrome is linked to germline BRCA1 or BRCA2 gene mutations. Mutations in other genes also involved in DNA damage repair and especially in Homologous Recombination (HR), like BRCA1 and BRCA2 genes, may also be associated with an increased risk of breast and ovarian cancers. Lynch syndrome is mainly characterized by a high risk of colorectal, endometrial and ovarian cancers and is linked to germline MLH1, MSH2, MSH6, or PMS2 gene mutations.

These two cancer predisposition syndromes are estimated to be involved in 15 to 20% of all OCs, with the contribution of HBOC at least tenfold greater than that of Lynch syndrome. These two syndromes are transmitted according to an autosomal dominant mode. At-risk women carry a monoallelic germline loss of function mutation of a responsible gene. However, the tumour presents somatic inactivation of the wild-type allele via a partial chromosomal defect (deduced from the observation of loss of heterozygosity at the gene locus) or via a point mutation or promoter methylation. Thus, according to Knudson's "two-hit" theory, although the predisposition is transmitted according to a dominant mode, its effect on the tumour is recessive.

2.2 Hereditary Breast and Ovarian Cancers

2.2.2 Identification of BRCA1 and BRCA2 genes and their molecular pathology

The BRCA1 and BRCA2 genes were identified in 1994 and 1995, respectively, after preliminary genetic linkage studies performed in breast cancer families [1,2,3] that allowed their chromosomal location. BRCA1 is located in chromosome 17 (17q21) and BRCA2 is located in chromosome 13 (13q12). Soon after localization of these genes, it was observed that families including at least one case of OC were more frequently linked to the BRCA1 locus than families with breast cancer cases only [4], thus highlighting that BRCA1 and subsequently BRCA2 genes are also OC-predisposing genes.

The BRCA1 gene has a coding sequence of 5,589 nucleotides distributed over 23 exons and the BRCA2 gene has a coding sequence of 10,254 nucleotides distributed over 26 exons. More than one thousand different loss of function mutations spread over large coding sequences has been reported to date in the various mutation databases. Most mutations are point or small mutations introducing a stop codon. Large gene rearrangements also account for 8.5% of BRCA1 mutations and 2% of BRCA2 mutations [5].

In addition to deleterious mutations, Variants of Uncertain Significance (VUS) are detected in about 8% of BRCA1 or BRCA2 analyses currently performed in breast and breast-ovarian cancer families. Considerable efforts, combining complementary approaches (epidemiological, genetic, functional studies), have been performed to characterize the significance of these variants [6]. These efforts are coordinated by the international ENIGMA consortium [7],which, in 2014, fused with the BRCA Challenge, the Global Alliance for Genomics and Health (GA4GH) designed to characterize VUS of all genes involved in genetic diseases. It is difficult to provide patients with suitable information regarding VUS because the geneticist must inform them that a VUS has been identified, that it cannot be currently used for genetic testing of his or her relatives, but that it could be used in the future.

Due to the complexity of BRCA1/2 molecular pathology in terms of analyses and interpretation and the law yield of positive tests in many severe breast and ovarian cancer families, suggesting that other genes may also be involved, sequential genetic testing of the family needs to be performed, as, whenever possible, full screening of the BRCA1/2 genes is performed in the individual most likely to be predisposed: a woman with a history of breast or ovarian cancer. When a deleterious mutation has been identified, a mutation-targeted test can be performed in the patient's relatives. Thus, in BRCA1/2-positive families, a negative result in a relative is reassuring.

2.2.3 Functions of BRCA1 and BRCA2 proteins, the homologous recombination pathway

The observation of the nucleus colocalization of the BRCA1 and RAD51 proteins during the cell cycle S phase was a breakthrough in the knowledge of BRCA1 function [8], as the amino-acid sequence of BRCA1 did not

provide any clues to a specific cellular pathway. BRCA1 and BRCA2 proteins are involved in the repair of DNA Double-Strand Breaks (DSB) by HR, a critical function for the survival of normal cells [9, 10, and fig 2.1]. In the absence of functional HR, unrepaired or incorrectly repaired DSBs lead to a massive loss of genetic information, genomic rearrangements or cell death. BRCA1 appears to have an early and broad role in the HR process via an ubiquitin ligase function: BRCA1 is involved in genome surveillance by the transmission and amplification of the signal induced by DSB; in addition, BRCA1 promotes HR via a modulatory role in the PALB2-dependent loading of BRCA2-RAD51 repair-machinery. Moreover, BRCA1 exerts negative control on the cell cycle, thereby allowing the cell to repair its DNA damages especially during S phase. Inversely, BRCA2 is directly involved in the HR process via the sequestration and release of small RAD51 recombinase molecules at the site of the DSB (fig.2.1). Biallelic germline BRCA2 mutations are responsible for Fanconi disease, a recessive disease with strong genetic heterogeneity, as 15 genes have been identified to date.

The involvement of BRCA1 and BRCA2 in DNA damage response (DDR) has led to the hypothesis that cells with a BRCA1 or BRCA2 defect could be more sensitive to alkylating agents that considerably increase DSBs and to molecules inhibiting DNA repair pathways other than HR, such as Base Excision Repair (BER) [11]. Poly-ADP-ribose-polymerase inhibitors are an emerging family of DDR inhibitors (see Chapter 13 J Ledermann).

As mentioned above, BRCA1 and BRCA2 mutations cannot account for all severe breast-ovarian cancer families, suggesting that other predisposing genes have yet to be identified. Consequently, partner genes of BRCA1 and BRCA2, especially those involved in the HR pathway, are good candidates to be associated with high cancer risks and higher tumour sensitivity to alkylating agents or DDR inhibitors in case of gene inactivation. Numerous genes have been tested in association studies. For example, CHEK2 and CHEK1 involved in cell cycle control, ATM, MRE11A, and NBN involved in the detection of DNA damage, and PALB2 and BRIP1 which are also Fanconi disease genes. Of note, no monoallelic deleterious mutation of RAD51 has yet been reported, probably because such mutations would be lethal. In contrast, mutations of RAD51 paralogs (duplicated genes during species evolution that have slightly diverged but still have very similar functions to those of the original gene) have been reported and are associated with an increased risk of ovarian cancer (see below). Most of these genes are currently at the stage of research and genetic testing has not been proposed to date.

2.2.4 Histology of ovarian carcinoma in BRCA1/2 mutation carriers

Lakhani et al. compared the pathological characteristics of 178 BRCA1 and 29 BRCA2 OCs to those of 235 age-matched controls [12]. Both BRCA1 and BRCA2 tumours were of higher grade than control tumours ($p<0.0001$ and $p=0.028$, respectively). Well differentiated and grade 1 tumours do exist in BRCA1/BRCA2 mutation carriers, but tend to be rare. Similarly to sporadic cases, papillary serous OC is the most prevalent type, observed in 44% and 48% of BRCA1 and BRCA2 mutation carriers, respectively, followed by the endometrioid type, 36% and 38% in BRCA1 and BRCA2 mutation carriers, respectively. The frequency of serous tumours is reported to be significantly higher among BRCA1 mutation carriers (OR: 1.84, 95%CI: 1.21-2.79), while the frequency of mucinous tumours is much lower (OR 0.13, 95%CI 0.05-0.34, $p<0.0001$). The distribution of histological types in BRCA2 tumours is similar to that in BRCA1 tumours, but not significantly different from the control distribution. The frequency of borderline tumours does not appear to be increased in BRCA1/2 mutation carriers. In the study by Zhang et al., no BRCA1/2 mutation was identified in a series of 112 cases of unselected mucinous carcinomas [13]. As in the general population, clear cell forms and carcinosarcomas are rare. In summary, BRCA1/2 OCs are classically poorly differentiated and of high grade, corresponding to the "type 2" pathway of ovarian carcinogenesis [14]. OCs in BRCA1/2 mutation carriers are thought to arise from serous intraepithelial tubal carcinoma (STIC) in the fallopian tubes, associated with TP53 somatic mutations [15].

2.2.5 Prevalence of BRCA1/2 and other HR pathway gene germline mutations among ovarian cancer cases

Before reporting the prevalence of BRCA1/2 germline mutations in women with OC, it is useful to recall the prevalence of these mutations in the general population (males and females). According to the Anglian Breast

Cancer Study, and taking into account the Hardy and Weinberg law, the allelic frequency for BRCA1 mutations in the general population was estimated to be 0.051% (95% CI: 0.021 – 0.125%) and the allelic frequency for BRCA2 mutations was estimated to be 0.068% (95% CI: 0.033 – 0.141%). The frequencies of BRCA1 and BRCA2 mutation carriers were therefore estimated to be 1/974 and 1/734, respectively. In other words, the frequency of BRCA1/BRCA2 mutation carriers in the general population is about 1/400 [16]. In the study by Song et al. described below, the observed frequency of BRCA1/BRCA2 mutation carriers among the 1,528 cancer-free controls was 0.37% (one BRCA1 mutation, 4 BRCA2 mutations), corresponding to 1/270 BRCA1/2 carriers in the general population, with 1/1,428 BRCA1 mutation carrier, and 1/333 BRCA2 mutation carrier [17].

Numerous studies have examined the prevalence of BRCA1/2 germline deleterious mutations in women with OC. The most recent studies performed in the largest series were based on a molecular testing approach that was as complete as possible [17, 18].

Song et al. performed germline analyses of BRCA1, BRCA2, MLH1, MSH2, MSH6, and PMS2 genes in a series of 2,222 women with invasive OC unselected for breast or ovarian cancer and in 1,528 controls. Proportions of histological subtypes: serous (57%), endometrioid (14%), clear cell (8.6%), mucinous (7.1%) and high grade (66%) were consistent with unselected OCs. Among the 2,222 OCs, 178 (8%) BRCA1/BRCA2 mutation carriers were identified: 84 BRCA1 mutation carriers (3.8%), 94 BRCA2 mutation carriers (4.2%). The proportion of BRCA1/BRCA2 carriers was higher in the high-grade subgroup, with 11% of carriers.

Alsop's study, conducted in a series of 1,001 consecutive cases of non-mucinous, non-borderline OC, identified 141 BRCA1/2 mutation carriers: 14.1% (95% CI: 11.9-16.3). About 2/3 of these cases were BRCA1 mutation carriers (88 cases) and 1/3 were BRCA2 mutation carriers (53 cases). In the serous and high-grade subgroups, 16.6% and 16.8% of cases were associated with a BRCA1 or BRCA2 germline mutation, respectively, and 17.1% of cases harboured combined characteristics. Notably, 45% of mutation carriers did not present a positive family history for breast and/or ovarian cancers, highlighting the fact that family history is not a sensitive marker for BRCA1/2 detection [18]. The indications for BRCA1/BRCA2 germline mutation testing in a patient with ovarian cancer are summarized in Table 2.3.

Walsh et al. used a high-throughput sequencing method to screen 21 BRCA1/2 partner genes that are candidates to be associated with an increased risk of breast or ovarian cancers in a series of 360 women with ovarian, peritoneal, or fallopian tube carcinoma. Mucinous carcinomas were excluded and a selection bias towards high-grade cases was observed, as 91% of tumours were high-grade. Among the 360 women tested, 24% carried a deleterious mutation: 18% in BRCA1 or BRCA2 (a figure similar to Alsop's study) and 6% in BARD1, BRIP1, CHEK2, MRE11A, MSH6, NBN, PALB2, RAD50, RAD51C, or TP53 [19].

2.2.6 BRCA1, BRCA2 and HR-associated genes: breast and ovarian cancer risks

Two meta-analyses examined the risk of breast and ovarian cancers in BRCA1 and BRCA2 carriers [20,21]. Note that the meta-analysis by Antoniou, performed without selection for family history, was included in the meta-analysis by Chen and Parmigiani, which combined both family and population-based studies and which mainly concerned the risks of breast and ovarian cancers (Table 2.1). Cumulative risks of ovarian cancer at ages 40, 50, 60, and 70 are reported in Table 2.2 [20]. The mean age at onset for both breast and ovarian cancers was younger in BRCA1 and BRCA2 carriers compared to the general population. In addition, according to the recent study by Alsop performed in a large series of OC women, the mean age at onset in sporadic cancer patients was 60.5 years versus 53.4 years and 59.8 years in patients with BRCA1 and BRCA2 mutations, respectively [18].

These figures correspond to mean cancer risks. Shortly after the identification of BRCA1 and BRCA2, it was observed that cancer risks may differ from one family to another (defined by close relatives) and among relatives of the same family. These differences were not chance differences, but were underpinned by modifying factors that can be either genetic or non-genetic, or by the nature/location of the causative mutation. Two international consortia have been established in order to identify such modifying factors: HBCCS and CIMBA. A recent study performed by CIMBA in a very large number of women (19,581 BRCA1 and 11,900 BRCA2 mutation

carriers) identified regions of the coding sequence in both genes in which the relative risk of ovarian cancer may be higher than the relative risk of breast cancer [22]. A genome-wide association study conducted on a series of 11,403,952 SNPs disseminated throughout the genome on 15,437 sporadic cases, 15,252 BRCA1 carriers, 8,211 BRCA2 mutation carriers, and 30,845 controls has also identified 6 SNPs associated with a slight increase of the relative risk of ovarian cancer. However, only two of these SNPs increase the risk of ovarian cancer in BRCA1 mutation carriers and only one increases the risk of ovarian cancer in BRCA2 mutation carriers [23]. The results of these extensive studies are disappointing at the present time, as they do not lead to any modification of the management of at-risk women, but they need to be pursued by combining factors of various origins.

Few data are available concerning ovarian cancer risk associated with germline mutation of genes involved in HR. At the present time, estimated cancer risks are only available for two RAD51 paralogs, RAD51D and RAD51C [24,25]. The relative risk of ovarian cancer was estimated to be 6.30 (95% CI: 2.86–13.85) in RAD51D mutation carriers and 5.88 (95% CI: 2.91–11.88) for RAD51C mutation carriers, which constitutes a >9% cumulative risk by age 80 [25, 26]. The lack of precise estimates of cancer risk associated with these newly identified genes is a major limitation to their use for genetic counselling in clinical practice. However, genes involved in HR could be used to guide treatment.

2.2.7 Prevalence of somatic inactivation of BRCA1/2 and HR genes in ovarian cancer and related diseases

Although it has been clearly demonstrated that, in the presence of a germline BRCA1/2 mutation, the second allele is somatically inactivated, identification of the BRCA1 gene immediately raised the question raised of its possible bi-allelic somatic inactivation. The article reporting the identification of BRCA1 in the October 1994 issue of Science was accompanied by another article reporting a study based on a series of 32 breast carcinomas selected for a deletion of the 17q arm, in which BRCA1 is located. Although three BRCA1 mutations were detected in the tumour, they all corresponded to germline mutations [27]. Consequently, up until recently, tumour inactivation of BRCA1/2 genes was considered to be mainly associated with germline mutations. However, recent studies based on high-throughput sequencing techniques in large series of ovarian cancers, have thrown new light on this issue , which is of critical importance with the recent development of DDR inhibitors, to which strictly somatically BRCA1/2 inactivated tumours are expected to also be sensitive.

The Cancer Genome Atlas (TCGA) project selected 316 high-grade serous ovarian carcinomas. Exome, promoter methylation, transcriptome, microRNA expression, and DNA copy number were studied for each tumour [28]. Germline DNA was matched. Tumour analyses identified 73 BRCA1/2 mutations (23%), which were of germline origin in 52 cases (17%). Conversely, in 21 (6%) tumours, BRCA1/2 inactivation was strictly somatic. In summary, 25% (21/73) of BRCA1/2 inactivations may be somatic. The BRCA1 promoter has also been shown to be methylated in about 10% of tumours, suggesting loss of expression. Genes of the HR pathway (EMSY, FANC, RAD51C, PALB2, CHEK2, BRIP1) have also been found to be mutated in the absence of BRCA1/2 inactivation.

In the study by Pennington et al., providing an update to the study by Walsh et al., 30 genes, including BRCA1, BRCA2 and 13 genes involved in the HR pathway and cell cycle control (BRCA1, BRCA2, ATM, BARD1, BRIP1, CHEK1, CHEK2, FAM175A, MRE11A, NBN, PALB2, RAD51C, RAD51D) were sequenced in a series of 390 cases of high-grade OC at both germline and tumour levels [19, 29]. A deleterious BRCA1/2 mutation was identified in 24% of tumours (18% germline and 6% strictly somatic). Deleterious mutations of other genes were also identified in 8.6% of cases (6% germline and 2.6% strictly somatic). The somatic/germline inactivation ratio was 25%, similar to that observed in the TCGA study (Figure 2.2). It is noteworthy that although germline HR pathway genes mutations do exist in low-grade serous carcinoma (11% of cases), no strictly somatic gene inactivation has been observed.

The Pennington study also reported that tumours demonstrating inactivation of the BRCA1/2 or HR pathway genes, regardless of its origin, are more sensitive to platinum-based therapy than non-mutated tumours [29]. Due to the complexity of genetic testing, especially on formalin-fixed, paraffin-embedded tissues, the availability of a tumour BRCAness or HRness signature would be highly desirable to select patients for clinical trials and

specific treatments. Such signatures, which correspond to genomic scars of the HR defect, are currently under development [30,31,32] and are starting to be used in clinical trials [33].

2.3 Lynch syndrome

2.3.1 Definition

Lynch syndrome, also known as Hereditary Non-Polyposis Colorectal Cancer (HNPCC), was first described by Henry Lynch, who reported rare familial aggregations of colorectal, gastric, endometrium, small bowel, biliary tract, urothelium tract, and ovarian cancer with early onset and whose distribution in one side of the family suggested a predisposing gene transmitted according to an autosomal dominant mode [34].The Amsterdam clinical criteria, initially defined arbitrarily in order to select families for identifying responsible genes, should now be abandoned. The current definition of Lynch syndrome is molecular, based on identification of an inactivating monoallelic germline mutation in a gene involved in the DNA MisMatch Repair pathway (MMR): MLH1, MSH2, MSH6, or exceptionally PMS2 [35]. As indicated for BRCA1 and BRCA2, Lynch syndrome is associated with a marked heterogeneity of deleterious mutations. In addition, there are also a large number of variants of unknown significance that require complementary classification studies.

2.3.2. Function of the MisMatch Repair pathway

The function of the MMR pathway is to correct DNA polymerase nucleotide misincorporations that may occur during DNA replication. Seven proteins compose the human MMR system with three MutS-homologs (MSH2, MSH3 and MSH6), and four MutL homologs (MLH1, MLH3, PMS1 and PMS2). MutS proteins recognise a mismatch, and recruit the ATP-bound MutL protein , then correct the mismatch. The MutS homodimer is formed by either MSH2/MSH6 (the MutSa complex) for single-base mismatches and short insertion–deletion loops or MSH2/MSH3 (the MutSb complex) for larger loops. The endonuclease function in the PMS2 subunit of MutLa (formed by MLH1 and PMS2) excises the DNA strand containing the wrong nucleotide and resynthesizes the excision gap via the replicative DNA polymerase.

In MMR pathway-deficient cells, short tandem repeat sequences, i.e. microsatellites, appear particularly prone to nucleotide misincorporations. The resulting MicroSatellite Instability (MSI) is a hallmark for MMR defects (for review, see 36). Lynch syndrome with genome-wide microsatellite instability therefore presents a signature of MMR dysfunction. This signature is applied in routine diagnosis. This signature is sensitive – the absence of MSI can almost formally exclude the diagnosis of Lynch syndrome (sensitivity of about 90%, but less reliable with MSH6) – and nonspecific, as MSI may result from MLH1 promoter methylation in late-onset colorectal cancers [37,38]. Techniques and interpretation are now well standardized. Immunohistochemistry (IHC) analyses of MMR protein expression should also be performed, as the mutated gene is expected to lead to loss of expression of the corresponding protein in tumour tissue, which may guide genetic screening [38].

Any case of Lynch syndrome spectrum cancer (see below) occurring before the age of 60 or even 70 years should be tested somatically for MMR system deficiency (deficient MMR phenotype - dMMR - defined by microsatellite instability - MSI - and/or loss of expression of MMR protein) (Table 2.3), as OCs can be considered to be like endometrial cancer, for which the combination of MMR protein expression followed by evaluation of MLH1 promoter region methylation in cases demonstrating MLH1/PMS2 IHC loss provided the highest positive predictive value for identification of mutation carriers in women younger than 60 years of age at diagnosis [39]. However, the current development of high-throughput sequencing techniques will radically change this stepwise diagnostic strategy by combining somatic prescreening analyses followed by germline MMR testing in selected patients. Nevertheless, these somatic analyses will still be useful, particularly for interpretation of the results and especially in the case of identification of VUS, rather than to define the indications for MMR gene screening.

2.3.3 Histology of ovarian carcinoma in Lynch syndrome carriers

Chui et al. performed a review of the published literature [40]. Among 168 ovarian carcinomas observed in Lynch syndrome patients, 54 (32.1%) were serous, 43 (25.6%) were endometrioid, 24 (14.3%) were clear cell, 14 (8.3%) were mucinous, and 33 (19.6%) were not otherwise specified, i.e., a n over-representation of non-serous ovarian cancers, such as endometrioid, clear cell, and mucinous carcinomas. Chui et al. then performed a centralized pathology review on 20 ovarian cancers from patients carrying a confirmed germline MMR mutation [41]. Surprisingly, this review revealed that all carcinoma were either pure endometrioid (14 cases, grade 1 or grade 2, no grade 3), mixed with an endometrioid component (4 cases), or clear cell (2 cases). No serous or mucinous carcinomas were identified in this small series. All tumours presented MSI. It should be noted that 19 of the 20 OCs were diagnosed at stage pT1 or pT2, consistent with low or intermediate grade, as Lynch syndrome-associated OCs result from type 1 carcinogenesis (TP53-negative, low-grade, [42]), but associated with a particular molecular profile, KRAS/BRAF non-mutated, and with a frequency of 30%, PIK3CA mutations, comparable to type 1 sporadic tumours [43].

2.3.4. Prevalence of Lynch syndrome among ovarian cancer cases

To our knowledge, few studies have examined the frequency of Lynch syndrome in the general population. Based on the results of MMR gene screening performed in two series of colorectal cancer cases combined with 1,044 Finnish cases, 2.7% of patients were MMR mutation carriers. Figures were extrapolated to estimates in the general population. The frequency of Lynch syndrome was estimated to be 1/740 in the general population [44]. In the above-mentioned study by Song, germline MMR mutations were identified in 5 out of 1,528 cancer-free controls tested for MLH1, MSH2, MSH6, and PMS2 germline mutations; extrapolation to the general population results in a prevalence of one carrier for 306 individuals [17].

Also in the study by Song, germline analysis of the MMR genes in a series of 2,222 patients with invasive OC identified a pathogenic mutation in 17 cases (0.76%), namely 10 MSH6 mutations, 4 MSH2 mutations, 2 MLH1 mutations, and one PMS2 mutation.

Pal et al. screened MLH1, MSH2, and MSH6 genes in a population-based series of 1,893 women with ovarian tumours, including borderline tumours (13.5% of the series) [45]. Nine deleterious mutations were identified in 9 individuals [0.5%; 95% CI: 0.2–0.8)], including 5 MSH6 mutations, 2 MLH1 mutations, and 2 MSH2 mutations.

Walsh et al screened 21 tumour suppressor genes, including MLH1, MSH2, MSH6, and PMS2, in a series of 360 women with primary ovarian, peritoneal, or fallopian tube carcinoma [18]. Cases of mucinous ovarian cancer were excluded. Most tumours (91%) were high-grade tumours. Only two deleterious germline MSH6 mutations were identified (0.5%), with no MLH1, MSH2, or PMS2 mutations. It is noteworthy that the only MMR gene found mutated in this series of cases selected for type 2 OC, while Lynch syndrome-associated OCs tend to be type 1, was MSH6. These results are consistent with those reported in the two previously cited studies, indicating that most patients with Lynch syndrome-associated OC were MSH6 mutation carriers.

In summary, Lynch syndrome patients represent a small proportion of ovarian cancer cases. Carrier frequency may be only about 1% and mutations involve the MSH6 gene in the majority of cases. However, Lynch syndrome should be suspected in any patient diagnosed with ovarian cancer before the age of 61 years and/or with a personal or family history of Lynch syndrome spectrum cancers (Table 2.3).

2.3.5. Cancer risks in Lynch syndrome

The "narrow cancer spectrum" of Lynch syndrome, defined by a relative risk higher than 8, includes colorectal, endometrial, urinary tract, and small bowel cancers. The "broad cancer spectrum", defined by a relative risk between 5 and 8, includes ovarian, stomach, and biliary tract cancers. Good estimates of cancer risks were provided by the ERISCAM study that was designed to avoid ascertainment bias in cases with a positive family history [46].

This study examined 537 individuals and their relatives with a germline mutation in one of the MMR genes [MLH1 (n = 248), MSH2 (n = 256) and MSH6 (n = 33)]. Table 2.4 reports cancer risks according to the gene identified. The specific ovarian cancer cumulative risk at the age of 70 years was estimated to be 8% (95%CI: 2-37%) in the entire study population. This risk was estimated to be 20% (95%CI: 1-65%) in patients with MLH1 mutation; 24% (95%CI: 3-52%) in patients with MSH2 mutation; and 1% (95%CI: 0-3%) in patients with MSH6 mutation (Table 2.5). Globally, the ovarian cancer cumulative risk at age 40 years was less than 1%. However, assessment of ovarian cancer risks is subject to caution in view of the small number of families, especially those with MSH6 germline mutations.

2.3.6. Impact of tumour microsatellite instability in the clinical management of patients

Survival with MMR deficiency has been extensively investigated in patients with colorectal cancer, but much less extensively in patients with ovarian cancer. The prognosis is definitely better with MMR deficiency, which can be explained by reactive immunity [47].The Microsatellite Instable subset in colorectal cancer seems to be a good immunotherapy checkpoint candidate [48]. Anti-PD-1 and anti-PD-L1 are new emerging therapeutic agents responsible for blockade of the Programmed Death (PD-1) pathway, a negative feedback system that represses the Th1 cytotoxic immune response. This pathway is upregulated in many tumours and blockade of this pathway by antibodies targeting either PD-1 or its ligands (PD-L1, PD-L2) has resulted in remarkable clinical responses. Some experimental and clinical data suggest that tumours with deficient-MMR (dMMR) phenotype may be more responsive to PD-1 blockade than proficient-MMR tumours (pMMR), as dMMR tumours have 10 to 100 times as many somatic mutations that have the potential to encode "non-self" immunogenic antigens, compared to pMMR tumours. dMMR tumours may therefore be more immunogenic and consequently more sensitive to these new immunotherapeutic approaches, as suggested by the results of the recently published phase II study by Le et al evaluating the activity of pembrolizumab, an anti-PD 1 immune checkpoint inhibitor, in a small number of pMMR colorectal cancers, dMMR colorectal cancers and in other dMMR cancer types (cholangiocarcinoma, endometrial, small bowel and gastric) [49]. If these results are further confirmed and extended to ovarian cancers, patients with sporadic or Lynch-associated dMMR ovarian cancers may benefit from administration of these new agents.

2.4 Cancer genetic testing issues: adding to prevention management and specific treatment choices

As mentioned in the introduction, the aim of genetic testing for "a cancer-predisposing gene" up until now has been cancer prevention in the tested individual and his/her relatives. A new era is opening with the advent of PARP inhibitors for the treatment of women with OC with BRCA1 or BRCA2 gene inactivation and, in the near future, for genes involved in HR. As up to 25% of BRCA1/2 inactivation may have occurred only in the tumour and may therefore be strictly somatic, it would be useful to start by testing the BRCA1/2 genes in the tumour. This new genetic testing issue and new testing strategies raise a number of challenges for molecular and clinical geneticists, oncologists, as well as patients. The resolution of these challenges may lead to a modification of current genetic testing practices.

Genetic testing is expected to increase in the future, as most women with ovarian cancer, including women with high-grade OC after 70 years of age, and their oncologists will systematically require BRCA1/2 genetic tests. Result delivery time will need to be shortened. Technical difficulties of BRCA1/2 full gene screening, including screening for large gene rearrangements on formalin-fixed, paraffin-embedded tissues, must not be underestimated. To avoid loss of opportunity, BRCA1/2 tests will probably need to be performed at both the germline and tumour levels.

Patients will be asked to consent to a test comprising multiple issues that are often difficult to understand. They will be required to give their consent at the time of diagnosis, associated with a high level of stress. Will patients really be able to provide their free and informed consent? Genetic testing starting with the tumour could be considered to dissociate genetic predisposition from therapeutic issues. However, even if the technical difficulties of tumour genetic testing are resolved in the near future, allowing tumour testing to be performed first, a positive

result, corresponding to the presence of a germline mutation in 75% of cases, will still constitute a cancer-predisposing genetic test. Patient information concerning genetic testing issues, support to help them communicate a positive result to their relatives, and their own personal psychological support will still be required.

Improvement of the interpretation of BRCA1/2 sequencing will remain of utmost importance, especially in terms of VUS. It is essential to enter VUS into specialized databases in order to contribute to their classification so that the patient can subsequently be informed once the significance of a VUS has been determined. As mentioned above, the international ENIGMA consortium and the BRCA Challenge of the Global Alliance for Genomics and Health are actively involved in this field. Maintenance of the participation of patients, oncologists, and geneticists in these initiatives constitutes a real challenge. Similarly, although new ovarian cancer genes have recently been identified as a result of high-throughput sequencing, precise estimates of cancer risk are impossible and screening tests cannot be performed in relatives in a diagnostic setting. Further epidemiological studies in patients and their relatives are required.

In summary, two principles must be taken into account for the definition of new cancer genetic testing guidelines: (1) patient information and support, (2) improvement of test quality, especially concerning interpretation of the results. More genetic counsellors specialised in cancer genetics are needed, oncologists must be educated about genetic testing issues and the difficulties of interpretation of the results, and new information media (phone, web, booklets) must be developed [50]. Epidemiological studies must be conducted and variant databases must be established. Networks between oncologists and clinical and molecular geneticists therefore need to be set up and will be a central component of these new guidelines.

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TABLES:

Table 2.1: 70-years cumulative risk of breast and ovarian cancer in BRCA1 and BRCA2 mutation carriers

Cumulative cancer risk at age 70	BRCA1 (95%CI)	BRCA2 (95%CI)
Breast cancer		
Antoniou et al, 2003	65% (44-78)	45% (31-56)
Chen & Parmigiani, 2007	57% (47-66)	49% (40-57)
Ovarian cancer		
Antoniou et al, 2003	39% (18-54)	11% (2.4-19)
Chen & Parmigiani, 2007	40% (35-46)	18% (13-23)

Table 2.2: Predicted ovarian cancer risk of a 30-year-old women carrying a BRCA1 or BRCA2 germline mutation (from Chen & Parmigiani, 2007,[21]).

Risk (%) of developing ovarian cancer by age				
30-year-old woman with a BRCA1/2 mutation	Risk at age 40 Mean (95%CI)	Risk at age 50 Mean (95%CI)	Risk at age 60 Mean (95%CI)	Risk at age 70 Mean (95%CI)
BRCA1	2.2 (1.6-3.4)	8.7 (6.7-12)	22 (18-27)	39 (34-43)
BRCA2	0.52 (0.28-1)	2.4 (1.5-4.2)	7.4 (5.1-11)	16 (12-20)

Table 2.3: Indications for molecular testing in women with OC

Indications for BRCA1/2 germline testing
Individual criteria: - Woman with ovarian cancer diagnosed before age 71 years (except for mucinous OC, borderline tumour, and non-OC) irrespective of family history - Woman with both ovarian and breast cancers, regardless of age at diagnosis
Family criteria: - Woman with ovarian cancer with (at least) one 1st degree relative (or 2nd degree if the link is a man) with breast or ovarian cancer, regardless of age at diagnosis.
Indications for screening for MMR gene germline mutations
- Woman with ovarian cancer diagnosed before 61 years - Woman diagnosed with ovarian cancer and a Lynch syndrome spectrum cancer (colon, rectum, endometrium, ovary, stomach, urinary tract, biliary tract, small bowel) regardless of age at diagnosis - Woman with ovarian cancer and a first-degree relative with a Lynch syndrome spectrum cancer (see above) - AND with MSI tumour (Lynch syndrome-associated ovarian or tumour) - OR in the absence of a somatic study with clinical features highly suggestive of Lynch syndrome and: familial aggregation of Lynch syndrome spectrum cancers concerning at least 2 generations and with at least one case diagnosed before the age of 50 years.

Table 2.4: Cumulative risks of cancers in Lynch syndrome for all genes (from Bonadona V, JAMA 2011, [46])

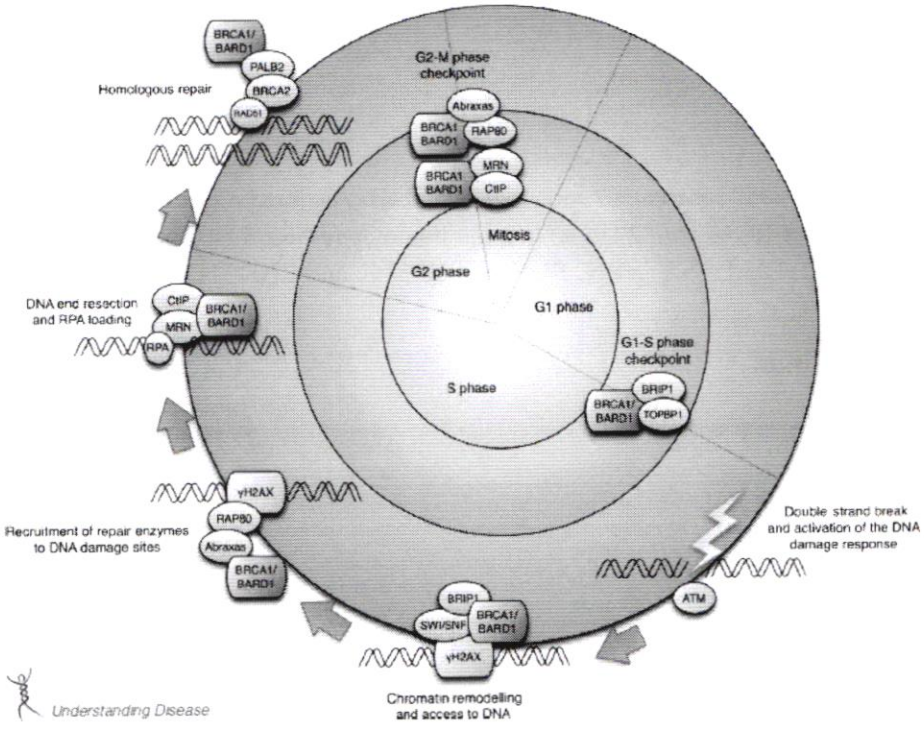
Cumulative risks of cancer at the age of 70 years	% (95%CI)
Colorectal cancer	35 (25-49)
Endometrial cancer	34 (16-58)
Ovarian cancer	8 (2-37)
Stomach	0.7 (0.08-4.4)
Urothelium	1.9 (0.3-5.3)
Small bowel	0.6 (0.1-1.3)
Biliary tract	0.6 (0.07-2.5)

Table 2.5: Age-specific cumulative risks of ovarian cancer according to genes for MMR mutation carriers (from Bonadona V, JAMA 2011,[46])

	Cumulative ovarian cancer risks			
Age (year)	All % (95%CI)	MLH1 % (95%CI)	MSH2 % (95%CI)	MSH6 % (95%CI)
30	0	0	0 (0-1)	0
40	1 (0-1)	0 (0-2)	1 (0-3)	0
50	3 (1-5)	4 (0-11)	4 (1-9)	0 (0-1)
60	7 (2-21)	15 (1-45)	11 (2-28)	1 (0-2)
70	8 (2-37)	20 (1-65)	24 (3-52)	1 (0-3)

FIGURES:

Figure 2.1: BRCA, DNA repair and the cell cycle (Foulkes and Shuen, 2013, [9])

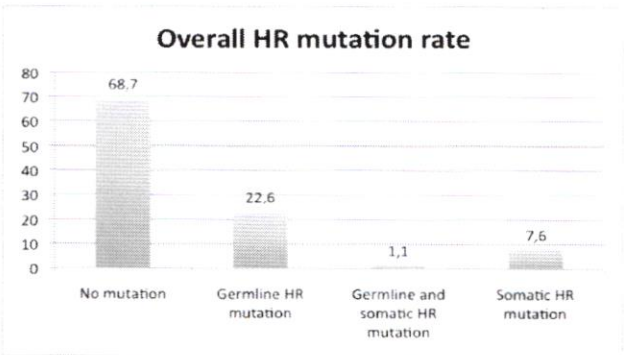


Legend of Figure 2.1: In response to DNA damage, BRCA1 mediates HR (depicted in the outer ring) and cell cycle regulation (depicted in the inner ring) when bound to different various macrocomplexes. Following a double-strand break, ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related) phosphorylate a number of downstream effectors, including H2AX, MRN (MRE11-RAD50-NBN), BRCA1 and its binding partner BARD1 (BRCA1-associated RING domain protein 1), initiating the DNA damage response (DDR). BRCA1 binds to BRIP1 (BRCA1-interacting protein 1) and SWI/SNF regulates histone deacetylases to open up the chromatin, perhaps allowing access of repair enzymes to the site of DNA damage. Following complex enzymatic modifications by ubiquitin and SUMO (small ubiquitin-like modifier), RAP80 (receptor-associated protein 80) and FAM175A (Abraxas) recruit BRCA1 and other downstream repair enzymes to the site of DNA damage. BRCA1, coupled with MRN and CtIP (C-terminal binding protein interacting protein), is involved in resecting the DNA ends to create single-stranded DNA (ssDNA), which is protected by RPA (replication protein A). The BRCA1/PALB2/BRCA2 macrocomplex is then required for RPA displacement

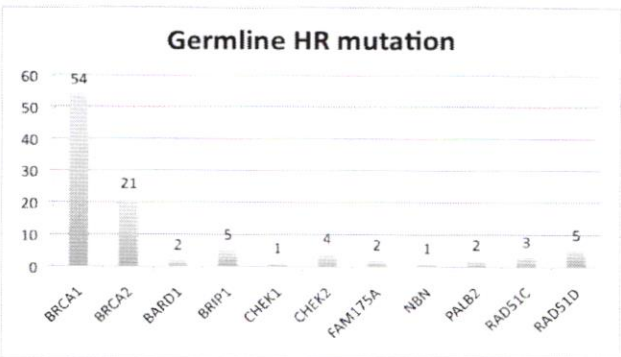
and RAD51 loading onto ssDNA. Finally, RAD51 mediates sister chromatid strand invasion and homologous repair. Acting in parallel with the DNA damage response are BRCA1 complexes that regulate the cell cycle. BRCA1 coupled with BRIP1 and TOPBP1 regulates G1 – S and intra-S phase checkpoints, while BRCA1/MRN/CtIP and BRCA1/RAP80/FAM175A (Abraxas) regulate the G2 – M phase checkpoint.

Figure 2.2: Mutation rates in homologous recombination (HR) genes (From Pennington et al. Clin Canc Res 2014, [29])

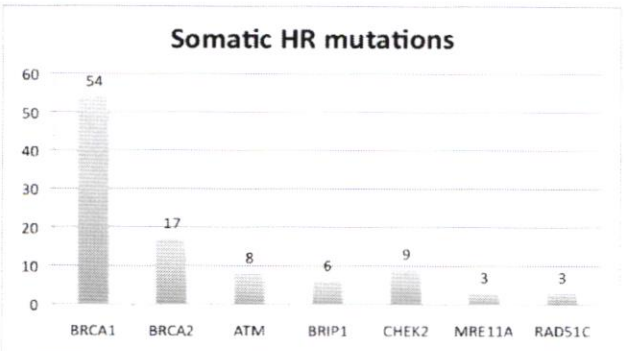
A. According to Pennington's study in 367 subjects, 115 (31.3%) had deleterious mutations in one of 13 HR genes tested: 83 (22.6%) with germline mutations, 28 (7.6%) with somatic mutations, and 4 (1.1%) with both germline and somatic mutations.



B. According to Pennington's study in 367 subjects, 87 subjects (24%) had germline mutations in 11 HR genes: 49 (13.4%) in BRCA1, 17 (4.6%) in BRCA2, and 22 (6%) in other homologous recombination genes, including BARD1, BRIP1, CHEK1, CHEK2, FAM175A, NBN, PALB2, RAD51C, and RAD51D.



C. According to Pennington's study in 367 subjects, 32 carcinomas (8.7%) had a total of 35 somatic mutations in 7 HR genes: 19 (5.2%) in BRCA1, 6 (1.6%) in BRCA2, and 10 (2.7%) in other homologous recombination genes, including ATM, BRIP1, CHEK2, MRE11A, and RAD51C.



WHOLE-EXOME SEQUENCING AND COMPARATIVE GENOMIC HYBRIDIZATION: COMPLEMENTARY APPROACHES IN ADVANCED GENOMICS

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Delay of motor development is a common diagnosis given in clinical practice to young children whose developmental milestones fail to be met in an age-related manner. Incidence reports that a diagnosis of developmental delay occurs in up to 15% of children under age five. Unfortunately, there is an increasing tendency over the last decade. Each year, approx. 40 thousand newborn are observed in Croatia, with numerous unresolved cases of motor developmental delay, which is a rather unsatisfactory situation for pediatricians, parents, and patients. A classical example for rare motor development delay has been discovered at the Zagreb Clinical Hospital Center in 2002 – S-Adenosylhomocysteine hydrolase (SAHH) deficiency. Since then, major milestones in disease characterization, genotype-phenotype relations, biochemical analysis, diagnostic evaluations and treatment planning have been achieved. However, several aspects of disease pathology remain unanswered. As same applies to numerous yet uncharacterized cases of motor development delay, and in order to provide answers to these questions, in particular, establishing a concise diagnosis, we have engaged in advanced genomics technologies such as Next-generation-sequencing (NGS). A particular interesting case observed in an infant was analysed by a combination of array based comparative chromosome hybridization (aCGH), and whole exome sequencing (WES) of mother-father-child trios. Results of this study, and lessons learned will be elaborated.

GENETICS OF NEUROCRISTOPATHY SYNDROMES: A WINDOW ON THE NON-CODING GENOME

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Disruption of long-distance highly conserved noncoding elements in neurocristopathies

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One of the key discoveries of vertebrate genome sequencing projects has been the identification of highly conserved noncoding elements (CNEs). Some characteristics of CNEs include their high frequency in mammalian genomes, their potential regulatory role in gene expression, and their enrichment in gene deserts nearby master developmental genes. The abnormal development of neural crest cells (NCCs) leads to a broad spectrum of congenital malformation(s), termed neurocristopathies, and/or tumor predisposition. Here we review recent findings that disruptions of CNEs, within or at long distance from the coding sequences of key genes involved in NCC development, result in neurocristopathies via the alteration of tissue- or stage-specific long-distance regulation of gene expression. While most studies on human genetic disorders have focused on protein-coding sequences, these examples suggest that investigation of genomic alterations of CNEs will provide a broader understanding of the molecular etiology of both rare and common human congenital malformations.

Keywords: malformation; genetics; neural crest; evolution; regulation

Introduction

One of the key discoveries of vertebrate genome sequencing projects has been the identification of highly conserved noncoding elements (CNEs). CNEs are predicted to occur at high frequency (3% when comparing human and mouse genomes, according to the ENCODE pilot project[1]). In particular, CNEs are enriched in gene deserts near key developmental genes.[2] A proportion of CNEs are potentially regulator elements (e.g., enhancers or silencers) influencing spatiotemporal gene expression during development.[3] While the maximum distance over which long-range regulatory elements can act is unknown, enhancers can be located over 1 Mb away from a gene.[4]

Historically, mutations that contribute to human malformation have been sought mostly in the coding regions of the genome (exons), whereas functional genomic elements independent of coding sequences and untranslated regions remain poorly investigated. However, a number of human developmental anomalies resulting from disruption of long-range regulatory CNEs have been reported, suggesting a potentially important role in normal growth, development, and disease.[5,6]

As a proof of principle, a variation in a CNE located in an intron of the *RET* proto-oncogene was recently identified as a frequent hypomorphic allele in Hirschsprung disease,[7] and deletions and a point mutation have been discovered in regulatory long-distance CNEs surrounding the *SOX9* gene in patients with Pierre Robin sequence (PRS), an endophenotype of a much more complex and lethal syndrome (campomelic dysplasia).[8] Both disorders feature among neurocristopathies and lead to the postulate that other congenital defects of neural crest-derived cells could be ascribed to mutations in long-distance regulatory CNEs. In this review, we summarize these findings and elaborate on their possible significance for other Mendelian disorders.

Defining conserved noncoding elements

The comparison of multiple genome sequences, now available across mammals and other species,

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revealed that in very distant species, both the number of genes and their coding regions were rather similar. Thus, great differences between distant species cannot be attributed to variations within coding sequences only. Conversely, the obvious difference between the simplest and the most complex organisms is the amount of noncoding sequence: the more complex the species, the greater the proportion of noncoding DNA, reaching about 98% in the human genome.[9] However, thus far, while the molecular bases of a great number of genetic disorders remain unknown, noncoding DNA has not attracted so much attention as a possible source of genome alteration in Mendelian disorders. Obviously, the limitations are not only technical, but also conceptual, since it is difficult to search for mutations in vast DNA domains without clues that might indicate their function. This is why conservation of sequences across species became the most obvious way to postulate that noncoding DNA might be functional, based on the assumption that evolutionary constraint for selection impacts on a DNA sequence, preventing genetic drift. Cross-species sequence comparisons would thereby potentially highlight noncoding DNA regions of putative biological importance (reviewed in Ref. 10). Those highly conserved noncoding elements (also reviewed in another chapter of the present volume) might be of great interest in genetic medicine, because, while their conservation might indicate function, their mutation might in turn be relevant to diseases in humans.

Screening highly conserved noncoding sequences for mutations is partly a blind approach, bypassing our ignorance of the function of those DNA segments, with the simple assumption that DNA variation located within them could impair their unknown biological properties. It goes without saying that, as for Mendelian disorders with high penetrance of mutant alleles, the usual criteria for significance of a DNA variant should also apply, such as (i) familial segregation according to the presumed mode of inheritance, (ii) *de novo* occurrence in a patient, and (iii) absence in controls from databases featuring single nucleotide polymorphisms. Further criteria for validation of a DNA variant occurring in a CNE would then involve both computational and biological investigations aimed at elucidating the function of the conserved sequence.

Comparative genome analysis, using mostly human and mouse genomes, demonstrated that evolution shapes the conservation of about 5% of the human genome, of which about 2% would contain protein-coding regions (exons and UTR sequences) and noncoding RNAs, whereas 3% of our genome would consist of conserved noncoding sequences.[11,12] Today, no particular nomenclature or anthology of those CNEs is defined, given that they can only be distinguished on a 2D rationale: length of the conserved sequences and depth of the conservation, that is, the evolutionary distance between the compared species. It is important to note that these CNEs do not define a family of sequences featuring particular motifs of primary DNA sequence. However, a family of ultraconserved elements has been defined according to criteria of 100% homology between human, mouse, and rat, over a distance greater than 200 bp;[13] for some of these elements, an enhancer (or possibly silencer) activity was demonstrated in reporter assays in embryonic mice.[3] Subsequently, a larger compendium of mammalian ultraconserved elements was identified.[14] The stringent definitions in these studies do not preclude the relevance of a more limited depth of conservation. A generally admitted, although disputable, threshold for qualification as a CNE would be sequences >100 bp with $>70\%$ conservation between human and mouse. In the case of regions of noncoding DNA with primate-specific functions, the disruption of which may result in behavioral or cognitive phenotypes in humans, the utility of cross-species comparisons as a means of highlighting functional elements may be limited, given the reduced evolutionary distance among primate genomes. The depth of conservation and the species included in the comparison may need tailoring for each genomic interval and phenotype studied in clinical genetics.

The functions proposed for CNEs fall into several categories. First, they may be regulatory elements involved in binding transcription factors, acting in *cis* with respect to the transcribed sequences. In this category, CNEs could activate or inhibit tissue-specific transcription, insulate regulatory regions, or participate within locus control regions involved in the concerted transcriptional regulation of a group of genes.[5] Such *cis*-regulatory CNEs could be localized at a very long distance to the coding sequence of their target gene, whose expression they regulate.

This raises the great difficulty of determining which gene is the target of a regulatory element. In the second category, CNEs may act as chromatin organizers. Indeed, about 8% of highly conserved CNEs (as described by Woolfe[2]) were found to bind SUZ12 (a core component of polycomb repressive complexes) in human ES cells, highlighting the role of CNEs in chromatin modification.[15] Third, CNEs could function as genomic regulatory blocks (GRB) dispersed throughout the genome in nongenic regions, gene-dense regions, or blocks of grouped genes. Finally, CNEs may function as regulatory elements acting in trans.[16]

Other hypotheses regarding the function of CNEs could be considered on theoretical grounds, including participation in the transcription or regulation of transcription of noncoding long interspersed transcripts, influence on the timing and origin of DNA replication, involvement in a higher order genome organization of chromosomes as far as their respective nuclear localization is concerned, and participation in the epigenetic phenomenon known as transvection, which results from an interaction between homologous alleles (reviewed in Ref. 17).

Impact on malformation of the neural crest

Neural crest cells

Neural crest cells (NCCs) form in the human embryo during the third to fifth weeks of pregnancy, within the neural folds that delineate the neural plate from the ectoderm. During the fusion of the neural folds, which ultimately yields a tube that will become the central nervous system, NCCs detach and become mesenchymal. They migrate throughout the body, integrating into nearly every organ.

NCC derivatives include the neurons and support cells of the entire peripheral nervous system (sensory and autonomic), adrenergic, and other endocrine cells, and all pigment cells except those arising from the retina (reviewed by Ref. 18). In the head, in addition to the cell types mentioned above, NCCs differentiate into connective and structural tissues such as dermis,[19] bones, and cartilage of most of the skull,[20] and muscle tendons.[21] They also infiltrate and are essential for the function of glandular and vascular elements such as the thymus, the thyroid and parathyroid glands, the conotruncal region of the heart, and the entire branchial vascular sector,[21–23] giving rise to connective, adipose, and smooth muscle cells.

The ultimate choice in phenotype made at a given site of differentiation is the result of a combination of extrinsic factors in the embryonic microenvironment and cell-intrinsic properties that modify the responsiveness of NCCs to these external influences. Both their migration pathways and fate are imposed on NCCs by surrounding tissues as they leave the neural primordium; these are less dependent on intrinsic properties regionally distributed along the neuraxis than had initially been presumed (reviewed in Ref. 18). For instance, truncal NCC transplanted at the vagal level colonize the gut and differentiate into enteric ganglia in which neurons synthesize acetylcholine rather than catecholamines, as they would have done normally. On the other hand, the cephalic NCC population contributing to the branchial arch-derived facial skeleton has some intrinsic positional information and commitment,[24] while the rostral endoderm imparts signals to the adjacent NCCs and thereby patterns craniofacial cartilages;[25,26] these studies highlight the importance of cross-talk between NCCs and their environment.

Definition of a neurocristopathy

Abnormal migration, differentiation, division, or survival of NCCs leads to highly diverse clinical and pathological features. Referring to their proposed common embryological origin, Bolande first introduced the concept of "neurocristopathy" in order to highlight the potential for shared pathogenetic mechanisms in the fields of both congenital malformations and tumor predisposition.[27]

Classification as a neurocristopathy has evolved from a "purist" definition to a larger view, including disorders involving tissues composed of both NCC and non-NCC-derived cell types, such as DiGeorge syndrome.[28] Additionally, endocrine, melanocytic and PNS tumors arising from NCC cell types have been described; these can be isolated or associated with anomalies of other derivatives of the neural crest (i.e., Multiple endocrine neoplasia type 2 A and B) or non-NCC cell types (i.e., Congenital central hypoventilation syndrome).

CNE variation within a gene: Hirschsprung disease

Hirschsprung disease (HSCR), or aganglionic megacolon, is a congenital malformation

characterized by the absence of enteric ganglia, which are NCC derived, along a variable length of the intestine. HSCR has served as a paradigm for dissectible diseases with complex inheritance since the 1980s. Indeed, it is frequent (1/5,000 live births), is diagnosed mostly in the neonatal period by objective histological criteria, and heritability is high (200).[29] Expressivity is an objective criterion that depends on the length of the aganglionic segment and is classified into short-segment (SS), long-segment (LS), and total colonic aganglionosis (TCA) forms. Among sporadic cases (80% of the cases), SS-HSCR is the most frequent (80%), and the sex bias is high (5.5/1) in favor of females. Conversely, familial cases are less frequent (20%), with both vertical and horizontal transmission, and the more severe the disease (LS and TCA), the more even the sex ratio. Therefore, the mode of inheritance is complex and sex dependant with a recurrence risk in sibs of 4% but with great variability (from 1 to 33%) when considering the gender, segment length, and familiarity of the index case. Indeed, according to Carter's paradox, the less frequent the presentation (i.e., a female with LS-HSCR), the higher the recurrence risk in sibs (reviewed in Ref. 29). Epidemiological studies favored an autosomal dominant, sex-dependant mode of inheritance in LS-HSCR, while autosomal dominant and autosomal recessive modes of inheritance were equally likely in SS-HSCR.[30]

The major disease-causing gene is *RET*, which encodes a tyrosine kinase receptor.[31] HSCR is caused by *RET* loss-of-function, due to large deletions, indels, frameshift, nonsense, and missense mutations scattered along the coding sequence of *RET*, with no mutational hot spot. A low-coding sequence mutation detection rate became rapidly obvious, because *RET* mutations were only identified in 50% of familial cases and 20% of sporadic cases.[32] Two sets of experiments were then performed: linkage analysis in vertical familial cases with at least three generations and transmission disequilibrium test (TDT) in familial cases with recurrence in sibs. Modifier loci were mapped at 9q31, 3p21, and 19q12.[33,34] Importantly, both experiments also showed that the impact of a genetic event at the *RET* locus was underestimated. Indeed, the vast majority of vertical and horizontal familial cases were compatible with linkage to *RET*, and almost all affected sibs shared at least one identical allele by descent at *RET*.

During the same period, overtransmission of a silent polymorphism encoded by *RET* exon 2 in sporadic cases had been observed by several groups (rs1800858, c.135G > A; p.A45A, reviewed in Ref. 29). Interestingly, the frequency of the mutant allele varies with the incidence of the disease in various ethnic groups; it is rare in Africa (about 1%), frequent in Europe (24%), and reaches 45% in Asia. The A allele falls within a 30 kb haplotype lying between the *RET* promoter and IVS5. The first intron of the *RET* gene is 23 kb and remarkably poor in regions conserved when comparing human and chick, with a unique conserved sequence of 250 bp at 9.7 kb downstream of the transcription start site, denoted MCS+9.7[7] (Fig. 1). A SNP (rs2435357) lies in the conserved sequence and the maximum transmission disequilibrium is found for the mutant T allele in HSCR patients.[7] In transgenic reporter mice, the sequence recapitulates the pattern of *RET* expression and can therefore be regarded as a target for binding tissue-specific protein(s).[35]

Recently, it has been demonstrated that MCS+9.7 binds SOX10,[36] a key transcription factor in NCC development that leads to Waardenburg syndrome (WS) type IV and II when mutated (reviewed in Ref. 37). Moreover, MCS+9.7 is the most powerful enhancer of reporter gene expression among all conserved sequences at the *RET* locus tested in luciferase reporter assays in a neuroblastoma (NB) cell line.[35]

Interestingly, two polymorphic SNPs (rs2435357 and rs2506004) in almost complete disequilibrium are found on the enhancer element. The dissection of the respective roles of each SNP is a difficult task (see Section 4). However, one or the combination of both mutant alleles is a low-penetrant predisposing allele to HSCR (hypomorphic allele) and the most frequent molecular event at the *RET* locus in cases presenting the common form of the disease, that is, males with nonfamilial SS-HSCR.[7,36]

HSCR is observed in combination with other congenital malformation(s) in 20% of neonates. Some are poorly defined associations and some may occur by chance, but others are well-characterized syndromes with Mendelian inheritance and with a growing number of known disease-causing genes.[29] Interestingly, the penetrance of the HSCR trait is extremely variable, ranging from 3–5% (Down syndrome) to over 80% (WS IV with *SOX10* mutation). These observations point to a putative role

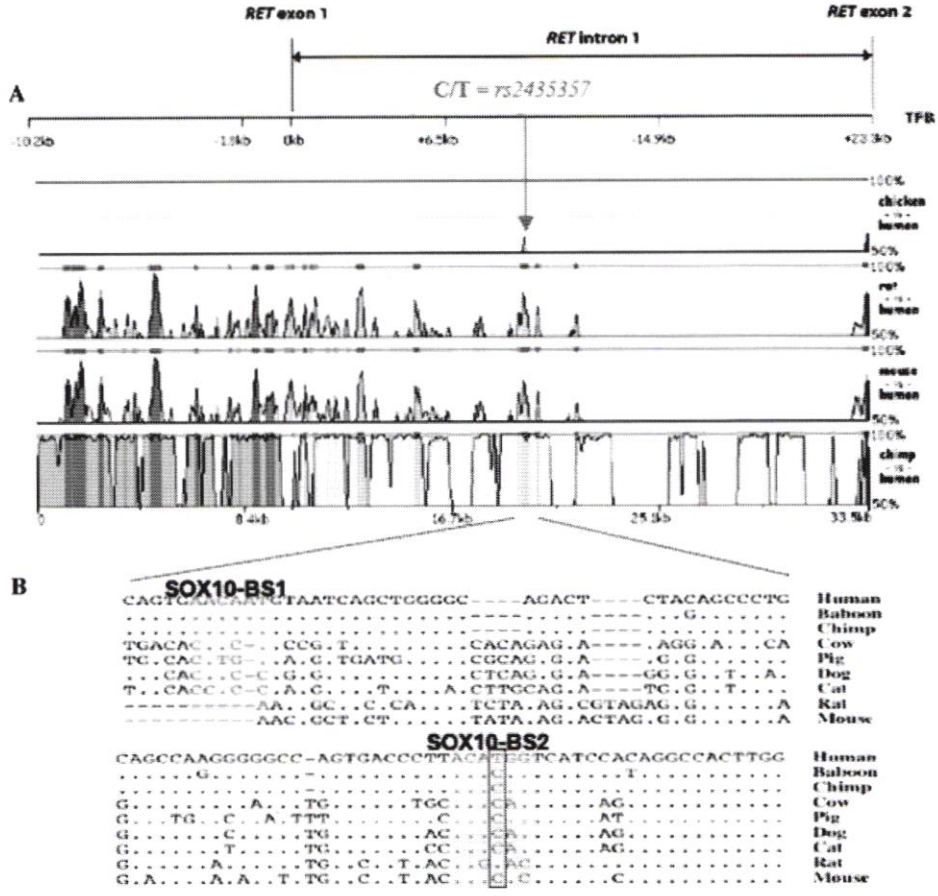

Figure 1. Overview of the sequence conservation within the first intron of *RET* (A), and the position of an HSCR-associated variant within the intronic CNE, MCS+9.7 (B). In A, graphs depicting conservation between human and other vertebrates were obtained from the ECR browser (http://ecrbrowser.dcode.org/). The position of MCS+9.7 is indicated by a red arrow. In B, a portion of the MCS+9.7 sequence is aligned with the corresponding region in other mammals, and predicted SOX10 binding sites (SOX10-BS1 and SOX10-BS2) identified by Emison *et al.*[36] are highlighted in yellow. The HSCR-associated C/T variant (boxed) falls within SOX10-BS2. Reproduced from Emison *et al.*[36]

of modifier loci for the HSCR traits for each syndrome. An obvious candidate is *RET*, which can be easily tested by genotyping the hypomorphic allele on IVS1 in all predisposing syndromes for which the gene (or chromosome) is known and a large enough series of affected patients with or without HSCR can be gathered. Thus far, such analysis had been carried out for Down, congenital central hypoventilation, Bardet-Biedl, Mowat-Wilson, and Waardenburg IV syndromes. The latter two were classified as RET-independent as the frequency of the hypomorphic allele was similar in the groups with and without HSCR for the same syndrome while the three others were found RET-dependent due to a significantly greater frequency of the hypomorphic allele in the group with HSCR when compared to the group without.[38,39] Interestingly, Bardet-Biedl syndrome being *RET*-dependent for the HSCR trait points to the role of cilia and the SHH signaling pathway for the normal development of the enteric nervous system.[40] Finally, these findings illustrate how the same gene can be both a major and a modifier gene depending on the genome.

A 4/1 biased sex ratio in isolated HSCR is suggestive of an X-linked modifier gene. However, neither linkage analyses nor TDT and case-control studies have pointed to the X chromosome. The only X-linked syndrome predisposing to HSCR is

MASA, which is due to mutation in the *L1CAM* gene. No *L1CAM* gene mutation was found in isolated HSCR.[41] Along the same lines, we genotyped seven SNPs with an informativity above 20% scattered along the *L1CAM* gene and found similar frequencies in 90 males with HSCR compared to controls (personal unpublished data). Interestingly, the upregulation of L1cam by Sox10 has been shown *in vitro* and loss of *L1cam* increases the incidence of aganglionosis of $Sox10^{+/lacZ}$ male mice.[42] A role for *RET* itself has been proposed based on the observation that among sib pairs the shared allele was significantly more often maternal.[7,34] However, the International HSCR Consortium data showed that 87% of the *RET* alleles in HSCR patients encode a loss-of-function protein when pooling the hypomorphic *RET* allele and coding sequence mutation alleles and therefore favoring an autosomal recessive mode of inheritance.[36] Interestingly, a biased sex ratio is also observed in *RET*-independent autosomal syndromes predisposing to HSCR.[39] This argues for genetic factors unlinked to *RET*. A possible explanation to the missing X-linked locus in HSCR could be that predisposing allele(s) have a frequency sufficient for homozygote mothers to be masking the locus. Indeed, a predisposing allele with a frequency of 70% would result in 50% of the females being homozygote and 70% of the males being hemizygotes. Interestingly, the penetrance of a *RET* CDS mutation is indeed of 70% in males and 50% in females. Altogether, the sex bias observed in HSCR remains largely unexplained at present.

Pierre Robin sequence

Neurocristopathies affecting the cardio-cephalic pole often leave their most visible mark on the face, since most facial tissues (bone, cartilage, teeth, vascular walls, and dermis) are direct NCC derivatives.

The Pierre Robin anomaly is regarded as a sequence since it combines three features that might result one from another as a cascade: micrognathia (mandibular hypoplasia) or retrognathia, leading to glossoptosis, itself preventing midline fusion of the palatal shelves and leading to a posterior U-shaped cleft palate (reviewed in Ref. 43). Pierre Robin sequence patients also feature functional anomalies mostly corresponding to hindbrain dysfunction: central apneas, sucking and swallowing defects, esophageal reflux, and cardiac rhythm dysregulation.[44] There is still an open debate on the embryological bases of PRS, that is, whether it can be regarded as a craniofacial neurocristopathy resulting from an abnormal development of the first branchial arch, or whether originating in an abnormal patterning of the hindbrain.

Based on a large series of 110 consecutive cases (1987–1996), it was apparent that PRS patients could present with other congenital malformation(s) in 52% of cases or could have isolated PRS (48%).[45] Among those cases with associated anomalies, several syndromes are recognized including a number of chondrodysplasias, branchial arch anomalies, neuromuscular disorders, or embryofoetopathies. Interestingly, in the chondrodysplasia group, the most frequently observed syndromes are Stickler and related syndromes (ascribed to anomalies of the genes encoding collagens 2A1, 11A1, and 11A2) and campomelic dysplasia, ascribed to coding sequence mutation of the *SOX9* gene on chromosome 17q24 (MIM114290).

When isolated, PRS cases are mostly sporadic. However, 13% are familial with a mode of inheritance that is most likely autosomal dominant with incomplete penetrance and variable expression.[45] Based on such familial cases, a PRS locus was mapped by a traditional linkage approach to chromosome 17q24.2-q25.1 in a large four-generation PRS family and later confirmed in smaller families.[8] Interestingly enough, that mapping region centered on the *SOX9* locus in the middle of a vast gene-desert, encompassing about 2.5 Mb of DNA (Fig. 2). However, no coding sequence mutation of the *SOX9* gene (nor of the flanking genes) could be detected in PRS cases linked to 17q24. Several observations of reciprocal translocations, for which one of the breakpoints in each case maps to 17q24, have been reported in families segregating PRS as an isolated trait.[8,46] Remarkably, all translocations clustered in a small DNA region, 1.2Mb upstream from the *SOX9* coding sequence. These data suggested that a genomic alteration responsible for the PRS phenotype might not impair coding sequence, but, rather, noncoding DNA. Comparative genomic alignments had previously indicated that the *SOX9* gene-desert is packed with CNEs conserved between humans and other mammals and, in some cases, between mammals and fish.[47] Comparative genomic hybridization and sequencing of candidate elements resulted in the discovery of several deletions and a point mutation involving or located within CNEs in

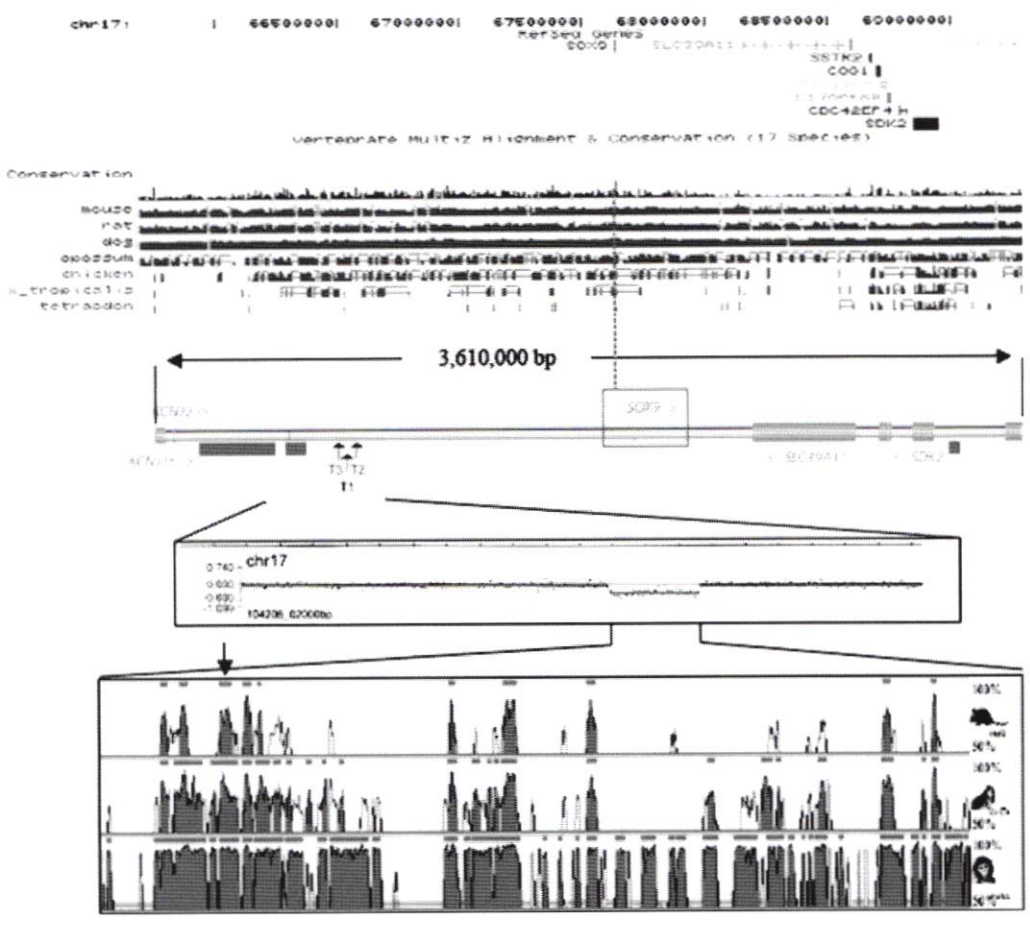

Figure 2. Distribution of CNEs within the gene desert surrounding *SOX9* and noncoding lesions identified in isolated PRS patients. The Multiz alignment was obtained from the UCSC genome browser (http://genome.ucsc.edu/). Beneath the alignment, the position of translocation breakpoints identified in Benko *et al.*[8] and Jakobsen *et al.*[46] are indicated by T1–3, and deletions identified in Benko *et al.*[8] are indicated with red boxes. In the lower half of the figure, the CGH data for one of the familial deletions is depicted, as is the distribution of conserved elements within the deleted region. A black arrow indicates the position of a point mutation identified within a CNE in a family with isolated PRS. Reproduced from Benko *et al.*[8]

sporadic and familial PRS cases mapping to 17q24 (Fig. 2), with either inherited or *de novo* occurrence of those DNA variations.[8] The wild-type versions of some of these CNEs, located at a long distance from the *SOX9* coding sequence, were able to drive reporter gene expression in the branchial arches of transgenic mice, suggesting they can indeed function as enhancers. The CNE sequence variation identified in a PRS family affected the binding of a transcription factor important for craniofacial development, MSX1. While the genetic data involving alteration of long-distance CNEs in PRS are overwhelming, the difficult question to address is the identity of the gene whose expression is impaired by such noncoding genomic alterations located deep in the middle of a gene-desert. Several lines of evidence strongly point toward *SOX9*, although definite proof may require further 3-C experiments or the generation of a mouse model whereby a targeted CNE deletion or mutation segregates with a PRS-related phenotype. There are several other arguments that support *SOX9* as the target of the enhancer(s) disrupted in the genomic lesions reported in.[8] The *SOX9* gene is conserved in all gnathostomes, and *SOX9* expression is conserved in the branchial arches of several animal models (and in the human embryo). *Sox9* is involved in early NCC development and in mandibular chondrogenesis,[48–50] while

cleft palate occurs in mice heterozygous null for *Sox9* and in mice with a conditional knockout of *Sox9* in NCCs.[48,49] The *SOX9* coding sequence is mutated in campomelic dysplasia, a frequently lethal syndrome combining bone dysplasia, sex reversal, and PRS in more than 80% of cases.[51,52] SOX9 functions as a transcription factor, activating the expression of the *COL2A1* gene, whose mutations result in a group of syndromes featuring PRS as an endophenotype.[53,54] Finally, the conformation of chromatin across a broad domain encompassing the *SOX9* locus, as shown by interphase FISH experiments,[8] is consistent with long-range interactions between the PRS locus and *SOX9*, and ChIP analysis in mouse cell-lines derived from the mandibular arch demonstrated the association of the PRS locus with markers consistent with a regulatory function of the locus.[8]

It was thus proposed that the long-distance genomic alteration in PRS patients may represent a site- and stage-specific loss of transcription, resulting in a restricted reduction of *SOX9* expression in human cranial NCCs and/or mandibular arch (reviewed in Ref. 55). This fits well with the tissue-specific regulatory nature of the CNEs within the *SOX9* gene-desert. Finally, these findings may be more broadly applicable, whereby the disruption of highly conserved sequences might explain the effect of genomic alteration in *cis* at long distances up or downstream of disease gene loci.

Other examples

A number of diseases or malformations have been ascribed to genomic alterations, located in noncoding DNA, sometimes at a very long distance from gene coding sequences. These conditions have been reviewed elsewhere,[6] including in the present volume, and the most relevant examples are listed in Table 1. It is remarkable that a number of genomic alterations involving CNEs are duplications; this could be an emerging theme in the molecular investigation of congenital malformations. Indeed, duplications may lead to a tissue-specific modification in gene dosage because of an increase in the number of enhancers or silencers lying in the duplicated segment. Duplications could also result in a general disruption of the chromatin conformation.

Position effects

Correct spatiotemporal expression of developmental genes requires the concerted action of a vast number of regulatory elements, located in *cis*, upstream or downstream of target gene coding sequences. These elements could be schematically involved in two processes: (a) direct control of gene expression via the binding of transcription factors and thereby recognized as silencers, enhancers, or promoter regions; or (b) higher order regulation of genome structure, such as chromatin organization.

Indeed, a number of possible mechanisms have been proposed to explain position effects of chromosomal rearrangements at considerable distance from the coding sequence of a known locus, including separation of the gene from an enhancer or promoter, juxtaposition with a heterologous enhancer in a novel chromosomal environment, removal of a long-range insulator, competition by a heterologous gene for an enhancer that would normally interact with the disease gene, and position-effect variegation resulting from spread of heterochromatic DNA.[6,56]

Implication for gene identification and mechanisms of disease

It is likely that the long-range control of gene expression is a concordant feature of a limited number of genes involved in multiple tissues at several stages of development. These genes, many of which may be transcription factors, are predicted to feature a highly complex regulation of their expression pattern, both spatially and temporally.

Implication for Mendelian disorders: a model

Given that the location of CNEs at a distance from the gene coding sequence challenges the definition of a gene, the domain of DNA to investigate in Mendelian disorders might be much broader than traditionally investigated and difficult to limit, possibly extending beyond neighboring gene(s). Since master developmental genes tend to lie in gene-deserts enriched in CNEs,[2] it is tempting to hypothesize that these are candidate regions for high-throughput DNA screening in congenital malformations in humans. Importantly, the PRS model may suggest that the alteration of long-distance noncoding sequences might result in endophenotypes, each of which would depend on the loss of tissue-specific expression that is driven by the cognate CNE, and the sum of which would recapitulate the complete syndrome phenotype ascribed to loss-of-function coding sequence

Table 1. A selection of human disorders ascribed to disruption of long-distance CNEs

Gene locus	Phenotype	CNE mutation	CNE function	Distance to CDS (kb)	Reference
SHH	Preaxial polydactyly	Point mutation/ duplication	Limb-specific enhancer (ZRS)	1,000	4
SHH	Holoprosencephaly	Point mutation	Brain-specific enhancer	460	69
RET	Hirschsprung	Point mutation	Intragenic enhancer	9.7	7
IRF6	Cleft lip	Point mutation	Enhancer	14	67
BMP2	Brachydactyly A2	Duplication	Enhancer	110	70
SOX9	Pierre Robin	Deletion/point mutation	Enhancer	up to 1,450	8
SOX9	Brachydactyly-anonychia	Duplication	?	~1,200	71
FOXL2	BPES	Deletion	Enhancer	280	72, 73
POU3F4	Deafness	Deletion	Enhancer	900	74
PAX6	Aniridia	Translocation	Enhancer	~150	75
SHOX	Leri-Weill dyschondrosteosis	Deletion	Enhancer	~250	58

Note: ZRS, zone of polarizing activity regulatory sequence; BPES, blepharophimosis ptosis epicanthus inversus syndrome. A question mark indicates uncertainty regarding the identity of the regulatory element disrupted.

mutations (Fig. 3). Such a model could be tested for major developmental genes for which a syndrome is ascribed to coding sequence mutations. The disruption of CNEs in human disorders could occur by many processes, including mutation, deletion/duplication and translocation, and should encourage fewer "coding-centric" studies.

Experimental attempts to map and characterize functional regulatory elements

A number of techniques are available to identify and map active *cis* regulatory elements, such as DNase hypersensitive mapping and ChIP experiments for the detection of protein-DNA interaction and histone modification. Besides those approaches, the regulatory potential of conserved sequences can be tested by *lacZ* reporter assays in transgenic animals such as zebrafish or mouse.[2,3] In addition to these traditional reporter models, transient transfection of enhancer-reporter constructs in chick embryos has enabled rapid screening of putative enhancers within a genomic region of interest, such as those at the *SOX2* and *SHOX* loci.[57,58] Indeed, the ease of electroporation of neural crest in the chick makes this cell type particularly suitable for enhancer analysis, as demonstrated recently for a novel *SOX10* enhancer.[59] Targeted disruption or mutation of the CNE of interest can be performed in various animal models although redundancy of regulatory elements/protein binding sites may dilute phenotypes. 3C and derived techniques can also be appropriate given that relevant tissue/cell lines are available.

Genes influenced by noncoding genomic alterations

The models for normal long-range regulation by *cis* regulatory elements, modulating the expression of gene coding sequences, are numerous. Several proposals have been made, including a looping model and a facilitated tracking model.[5] Today, these models are difficult to address and distinguish experimentally, and could require innovative technologies. Indeed, the question of which gene (or genes) is influenced by the disruption of noncoding sequences remains challenging. In other words, while the proof of the importance of the genomic disruption in noncoding regions of the genome might be overwhelming (segregation of the lesion with affected individuals, *de novo* occurrence, absence from controls or CNV or SNP databases, sequence conservation across evolution, *in vitro* and *in vivo* demonstration of reporter expression consistent with regulatory element function), the identity of the gene or genes with which those CNEs interact might often remain elusive, especially as the regulatory elements can often be located more than 1 Mb upstream or

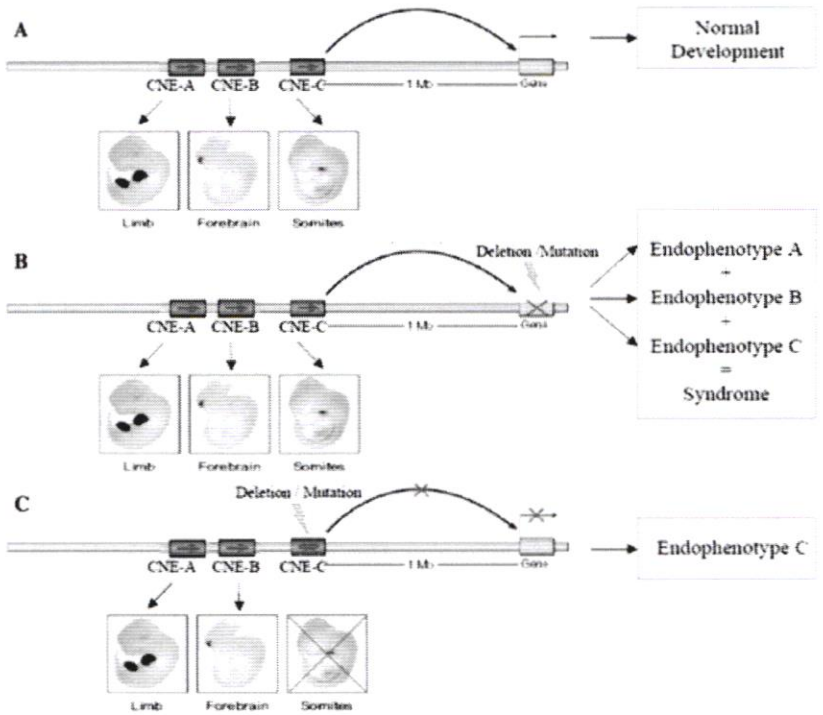

Figure 3. A model predicting the existence of endophenotypes of human syndromes caused by mutations in CNEs. Expression of key developmental genes requires the activity of tissue- and stage-specific CNEs located at a long distance from the transcription start site (A). Three examples of tissue-specific enhancers driving *lacZ* reporter expression in transgenic mice are illustrated. Mutation within the coding sequence of the gene causes a syndrome composed of several tissue-specific endophenotypes (B). A lesion disrupting a single CNE may result in an endophenotype caused by loss of expression of the gene in an isolated tissue (C). Images of transgenic mouse embryos are reproduced from Pennacchio *et al.*[3]

downstream of the gene coding sequences. In addition to the types of evidence listed above, other arguments may add weight to the likelihood that a candidate regulatory element targets a specific gene. The information to be considered could include the expression pattern and function of the candidate target gene, with respect to the disease phenotype; the identity of the transcription factors interacting with the mutated/deleted noncoding sequence, their relevance to the developmental events that are predicted to be disturbed in the disorder, and their hierarchical relationship to the candidate target gene; the general organization of the genomic regulatory blocs with respect to the gene coding sequence; and the general chromatin context.

Perhaps the best method for determining whether a particular gene is the relevant target of an enhancer is via targeted disruption of that enhancer in mice. Subsequently, expression analysis of the genes surrounding the deletion would potentially indicate the relevant in vivo target. Essential functions for several tissue-specific enhancers have been demonstrated by targeted deletion, including those at the *Emx2* and *Shh* loci.[60,61] In a recent example, a knockout of a noncoding interval, known to contain SNPs associated with coronary heart disease in humans, resulted in defects in vascular cells and affected cardiac expression of genes neighboring the interval; although discrete enhancers were not identified, this study provided strong evidence for the *cis*-regulatory influence of the noncoding region.[62] On the other hand, targeted deletion of predicted enhancers can result in mice with no obvious abnormalities.[63] This may be an indication of enhancer redundancy, or may be due to limitations of phenotypic screening. In contrast to reverse genetics techniques in mice, the identification of genomic alterations in noncoding DNA in humans with cognitive and behavioral phenotypes may be the most sensitive means of identifying elements that regulate gene expression in the mammalian brain.

Regarding the investigation of genomic interactions, a number of high-throughput techniques have been devised to investigate chromatin structure in detail over large genomic domains. In particular, ChIP experiments with microarrays as well as the 3C and derived techniques[64] are aimed at detecting specific patterns of modification that might allow a more global view of the organization of *cis*-regulatory elements across a particular region.

It is clear, however, that several other unknown phenomena might play an important role, which could be more difficult to investigate. Such mechanisms might involve the cross-talk between chromatin structure and noncoding RNA transcribed over large regions of noncoding DNA, the 3D nuclear architecture of chromosomes and gene loci as well as the spatial organization of the chromatin fibers in the interphase nucleus and the location of a gene relative to other chromosome territories.

Conservation of function without conservation of primary DNA sequence

It has been recently shown that some DNA regions might contain regulatory functions, without having a clear degree of conservation across multiple genome species. In particular, using transgenic reporter assays in zebrafish, it was shown that DNA sequences within noncoding regions, and with no conservation of the primary DNA sequence, could be functional.[65,66]

However, conservation of sequences, across the most divergent genomes possible, remains a good standard for deduction of function. A careful analysis of such sequences involves the length of the DNA in question, the depth of conservation and the proportion of consistent nucleotides across species. A number of bio-informatics tools are available to align such sequences. The question of the nomenclature of evolutionary conserved elements is still open.

Implication for common malformations and complex disorders

As suggested by the PRS mode, the alteration of long-distance noncoding sequences might result in a limited endophenotype of a full-blown syndrome phenotype ascribed to loss-of-function coding sequence mutations in a given gene. Importantly, it may open new routes to the molecular dissection of common malformations such as cleft lip and palate, neural tube defects, and congenital cardiac defects. In order to test this hypothesis, large series of patients presenting a single common malformation need to be investigated for alterations in a number of CNEs at master developmental gene loci, keeping in mind that disruption of *cis*-regulatory elements may even lead to phenotypes not observed within the full-blown phenotype caused by CDS mutation. This hypothesis already has a few examples, such as a SNP disrupting an AP2alpha binding site in a *cis* enhancer element of the *IRF6* gene and resulting in a higher risk for cleft lip.[67] Similarly, sequence variations in *cis*-regulator elements may underlie a significant proportion of disease-associated QTLs. One of the best known examples is SNP variants in *cis* regulatory elements of the lactase promoter gene allowing persistent high-level expression of lactase. A more recent example involves allele-specific chromatin remodeling at a locus associated with asthma and autoimmune disease.[68] The vast clinical consequences of variations in spatiotemporal regulation of coding genes are just beginning to emerge and should become as frequent as, or even more frequent than, coding sequence mutations.

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Conflicts of interest

The authors declare no conflicts of interest.

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GENETICS OF PROGEROID SYNDROMES

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As the world's population is rapidly aging, driven by sustained increase in lifespan, age-related diseases, in specific the increasing cancer rates represent a major challenge for health care systems. Monogenic syndromes with highly penetrant tumor susceptibility and/or signs of premature aging affecting more than one tissue have been instrumental in identifying genes and pathways involved in carcinogenesis and age-related diseases. The latter are commonly defined as segmental progeroid syndromes and can be caused by germline mutations in genes encoding DNA repair proteins with concomitant cancer predisposition. Examples include the Werner helicase gene (WRN) in Werner syndrome and the Bloom helicase gene in Bloom syndrome. Some progeroid syndromes are caused by mutations in nuclear lamina associated genes, e.g. lamin A/C (LMNA) in Hutchinson-Gilford syndrome or BANF1 in Nestor-Guillermo progeria. While LMNA mutations are also found in a few atypical cases of Werner syndrome, some patients with suspected Werner Syndrome do not harbor mutations in any known progeria gene. An overview on the genetics of progeroid syndromes, with special emphasize on the findings of our group including the lately identified novel genetic causes will be presented. The latter will include the Ruijs-Aalfs progeroid syndrome, caused by mutations in SPRTN, and Mandibular hypoplasia, Deafness, and Progeroid features (MDP) syndrome, caused by mutations in POLD1.

SPINAL MUSCULAR ATROPHY (SMA): FROM GENE AND MODIFIERS TO THERAPY

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Not only complex disorders but also monogenic diseases can be strongly modulated by further genetic, epigenetic or even external factors, sometimes leading to full protection, known as incomplete penetrance. Approaches including whole transcriptome, exome, genome, methylome or proteome analyses of highly discordant phenotypes can help to identify these modifiers. In this lecture I will present the complexity of the molecular genetic basis of SMA, the main modulator SMN2, independent modulators such as plastin 3, how plastin 3 helped us to unravel the main cellular processes disturbed in SMA, and the most advanced strategies and progresses made in SMA therapy.

What makes SMA unique?

1. Patients with SMA show a homozygous functional loss of the survival motor neuron 1 gene (SMN1), but all patients carry one or more SMN2 copies that modulate the severity of the disease and can be targeted by small molecules and drugs. 2) About 94% of all SMA patients carry the same type of mutation, which allows simple molecular genetic testing. 3) SMA carriers with frequencies varying between 1:8 and 1:105 among various populations can be easily and reliably identified by quantitative PCR or MLPA. The frequency in Europe is 1:35. 4) A duplication of about 500 kb on chromosome 5q13.2, including SMN, occurred late in evolution, in primates, while the two different SMN genes are human-specific. 5) The main functional difference between SMN1 and SMN2 is a single, translationally silent nucleotide exchange in exon 7 that affects splicing regulatory elements and causes exon 7 skipping in 90% of transcripts. 6) Both FL-SMN1 and FL-SMN2 transcripts encode an identical SMN protein - a finding that is essential for therapeutic strategies aiming to increase SMN expression levels. 7) Discordant families with large phenotypic discrepancies pointed towards factors/pathways able to circumvent the detrimental impact of reduced SMN levels on motor neurons. Modifiers such as plastin 3 (PLS3) have shown to fully protect against SMA in humans carrying a homozygous deletion of SMN1. This knowledge can be further used for the development of SMN-independent therapeutic strategies. 8) Despite ubiquitous SMN expression, reduced SMN levels mainly affect motor neurons in the ventral horns of the spinal cord. However, in severe SMA patients additional organs can be involved. These findings are essential for future therapeutic considerations. 9) Postnatal therapies using antisense oligonucleotides facilitating correct splicing or gene replacement therapies using virally transduced SMN1 rescued the phenotype in SMA mice, however, only when administered in the first three days of life. This emphasizes the importance of pre-symptomatic treatment and the development of neonatal screening programs. 10) Some drugs, such as HDAC inhibitors can only slightly improve the disease outcome but not rescue the SMA phenotype. Nevertheless, they might be essential to further maintain SMN2 expression during lifetime. 11) SMA can be considered as a developmental and neurodegenerative disorder in severely affected SMA patients and a neurodegenerative disorder in milder affected SMA individuals.

EXPERT OPINION

Moving towards treatments for spinal muscular atrophy: hopes and limits

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Spinal muscular atrophy (SMA), one of the most frequent and devastating genetic disorders causing neuromuscular degeneration, has reached the forefront of clinical translation. The quite unique genetic situation of SMA patients, who lack functional *SMN1* but carry the misspliced *SMN2* copy gene, creates the possibility of correcting *SMN2* splicing by antisense oligonucleotides or drugs. Both strategies showed impressive results in pre-clinical trials and are now in Phase II-III clinical trials. SMN gene therapy approaches using AAV9-SMN vectors are also highly promising and have entered a Phase I clinical trial. However, careful analysis of SMA animal models and patients has revealed some limitations that need to be taken very seriously, including: i) a limited time-window for successful therapy delivery, making neonatal screening of SMA mandatory; ii) multi-organ impairment, requiring systemic delivery of therapies; and iii) a potential need for combined therapies that both increase SMN levels and target pathways that preserve/rescue motor neuron function over the lifespan. Meeting these challenges will likely be crucial to cure SMA, instead of only ameliorating symptoms, particularly in its most severe form. This review discusses therapies currently in clinical trials, the hopes for SMA therapy, and the potential limitations of these new approaches.

Keywords: antisense oligonucleotide therapy, gene modifier, gene therapy, neonatal screening, neuromuscular disorder, pharmacotherapy, SMN1, SMN2, spinal muscular atrophy

Expert Opin. Emerging Drugs [Early Online]

1. Introduction

Spinal muscular atrophy (SMA) is a devastating neuromuscular disorder that leads to progressive muscle weakness and atrophy and that represents the most common lethal genetic disease in infants. Patients with SMA are divided into clinical subcategories (termed SMA type I, II, III and IV) based on disease onset and severity, with SMA type I having the earliest onset and most severe phenotype [1]. Although SMA is considered to be a motor neuron disorder, additional organs can also be impaired, albeit mainly occurring in severely affected SMA mice and patients [2].

SMA is caused by functional loss of *SMN1*, whereas disease severity is influenced by the number of *SMN2* copies and other SMA-modifying genes reviewed in [3]. As *SMN2* mRNA is mainly alternatively spliced lacking exon 7 due to a single translationally silent variant, 90% of SMN protein is truncated and unstable. The remaining 10% are full-length transcripts, producing a protein identical to that encoded by *SMN1* reviewed in [3]. As the SMN protein has a housekeeping function in small nuclear ribonucleoprotein biogenesis and splicing, the multi-organ impairment found in severely affected SMA mice or patients is an obvious consequence of SMN expression levels that fall under a certain critical threshold [2]. At present,

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there is no curative treatment available for patients with SMA, but impressive progress has recently been made towards the development of new therapies.

Here we discuss: i) current progress towards a therapy for SMA, and ii) potential limitations, based on novel biological observations in SMA animal models and SMA patients that will impact on the design and delivery of future therapies. In the expert opinion section we will discuss potential strategies to overcome these constraints.

2. Current progress towards SMA therapy

2.1 SMN-dependent therapies

The main focus of translational SMA research at present is the development of SMN-dependent therapies. These efforts include strategies directly targeting SMN protein stability, endogenous *SMN2* mRNA transcription, or splicing by using small molecules (antisense oligonucleotides, AONs) or drugs, and approaches based on SMN gene replacement using self-complementary serotype 9 adeno-associated virus vectors (scAAV9) expressing *SMN1*.

Indeed, a high increase in central and peripheral SMN levels, leading to neuromuscular and systemic improvements, have been reported in recent preclinical trials in SMA mice using intravenous injection of scAAV9-SMN [4-6], subcutaneous delivery of SMN_{Rx}-AONs [7], or orally delivered small molecules [8]. A first Phase II clinical study using intrathecal delivery of SMN_{Rx}-AONs targeting *SMN2* pre-mRNA in SMA type I patients showed some encouraging results, including increased muscle function scores [9]. Consequently, a first 2:1 randomized Phase III clinical trial in 117 SMA type II and III patients was launched by ISIS Pharmaceuticals (NCT02292537). Hoffmann La-Roche is currently recruiting 48 SMA patients (aged 2–55 years) in a double placebo-randomized Phase I study (NCT02240355) to test safety and tolerability of their orally applicable compound RO6885247 (former PTC RG7800) [8]. Novartis initiated an open label study (NCT02268552) to investigate their splice correction compound LMI070 in 22 SMA type I patients for safety, tolerability, pharmacokinetics and pharmacodynamics. Moreover, a gene therapy approach, using sc-AAV9-CB.SMN, entered the clinical phase at the Nationwide Children's Hospital in Ohio, USA (NCT02122952), to evaluate safety and efficacy. A 3-cohort phase I study (escalade dose) has been initiated in May 2014, involving 18 SMA type I patients. Taken together, these various studies should provide a robust overview of the promise (and potential pitfalls) of targeting SMN levels in patients with SMA.

Previous drug development efforts based around histone deacetylase (HDAC) inhibitors also deserve attention as they are the only ones to date that have completed Phase III clinical trials. Valproic acid (VPA), one of the first HDAC inhibitors, was shown to increase *SMN2* mRNA and SMN protein levels *in vitro* and *in vivo*. A first clinical study of VPA in 20 SMA type I, II and III patients demonstrated an increase of FL-*SMN2* levels in ~ 1/3 of patients the 'VPA-responders' [10], which seems to depend on CD36 expression, a fatty acid translocase [11]. Several open-label and placebo-controlled clinical trials with valproate and L-carnitine have been completed with only little significant phenotypic improvements in 2–5 year old SMA type II and III patients [12,13]. However, the placebo-controlled study lasted only 6 months, a too short time period to expect a significant outcome in this disorder. Currently, a Phase III randomized clinical trial is ongoing in India (NCT01671384), which includes 60 patients aged 2–15 years.

2.2 SMN-independent therapies

Several interesting SMN-independent pathways with the potential for therapeutic targeting in SMA have recently been identified. A clinical trial using one non-SMN targeted compound, the neuroprotective drug Olesoxime (TRO19622, Trophos), has shown modest improvements in motor function in SMA type II and III patients [14]. However, as the majority of non-SMN targeted drugs are still in pre-clinical phases of development, they will be considered in section 4 below.

3. Limitations of current SMA therapies

Although none of the above-mentioned approaches, except for those using HDAC inhibitors, have yet completed a Phase III clinical trial and showed substantial benefit for patients, there is well-warranted excitement and hope in the field, mainly based on the promising preclinical results. Importantly, however, none of the treatments currently progressing through human clinical trials detailed above are likely to offer a complete 'cure' for SMA. We suggest that three main confounding factors arising from the early and systemic nature of SMA will therefore need to be addressed in the next stages of therapy design and testing.

3.1 Therapeutic time window

Data from animal models of SMA provide strong evidence for the presence of a critical 'therapeutic time-window' for delivery of SMN-targeted therapies [15]. Indeed, increasing SMN levels after the onset of overt symptoms (postnatal day 8 in SMAΔ7 severe mouse models) provided only very little amelioration of disease symptoms [4,7] questioning the future efficacy of SMN-targeted treatments when delivered to symptomatic (particularly type I) SMA patients. This is of particular concern as there is currently no neonatal genetic screening for this disease, usually diagnosed after the appearance of the first clinical signs. Thus, SMN-targeted therapies delivered after disease onset may only have a limited capacity to ameliorate disease symptoms. In contrast, restoration of SMN levels in a milder SMA mouse model significantly improved motor abilities, underscoring a potential difference concerning the timing and nature of the therapeutic window [16] in severe versus mild forms of SMA [3].

3.2 Systemic nature of SMA

Given the growing awareness of the multi-organ nature of SMA (particularly in the most severe forms of the disease [2]), and the need to deliver therapies systemically in mouse models of SMA to achieve full benefit [7,16], standard drug-based pharmacological approaches offer a potentially attractive route to quickly develop effective, systemic disease-modifying therapies for SMA, which may be used either alone or in combination with molecular therapeutic approaches (including ASOs and AAV gene therapy).

3.3 Insufficient restoration of SMN levels and/or biodistribution using current SMN-targeted approaches

Although the first clinical trials using intrathecal injection of SMN_{Rx}-AON in SMA type I patients have reported some partial amelioration of disease symptoms, it is still uncertain whether the extent to which SMN levels have been increased will be sufficient to cure SMA. Measurements of SMN protein levels in cerebrospinal fluid after multiple dosing of SMN_{Rx}-AON showed a ~ 120 – 160% increase from the depleted levels observed in untreated patients [9]. A range of previous *in vivo* and *in vitro* studies suggest that these modest increases most likely will not be sufficient to turn an SMA type I patient into a healthy individual, but rather may only reduce the severity of the disease (e.g., 'convert' a SMA type I into a type II or III patient), even if therapy could be started pre-symptomatically. Moreover, SMA mice carrying two *SMN2* copies treated systemically and pre-symptomatically with SMN_{Rx}-AON or SMN-AAV9 remained smaller, showed reduced survival, and did not recover full muscle activity and body weight phenotype seen in wild-type control animals [4,5,7]. This means that additional functional support will be required to fully ameliorate disease pathophysiology in SMA.

4. Expert opinion

At present, there is no cure available for patients with SMA. One drug (Olesoxime, Trophos) and one AON (SMN_{Rx}, ISIS Pharmaceuticals) showed in Phase II – III clinical trials some encouraging results, but no major amelioration was observed. Additional drugs (RO6885247 from Roche and LMI070 from Novartis) are under clinical investigation. However, the long-term ambition of the SMA community remains to: i) substantially ameliorate SMA in patients after development of symptoms; and ii) fully counteract development of SMA in people with *SMN1* deletion by delivering effective treatments at critical points in the therapeutic time-window (likely to mean pre-symptomatic delivery).

Work on animal models has clearly demonstrated that early therapeutic intervention is mandatory to achieve the best protective effect [4-7]. For this to be translated to patients, *SMN1* deletion pre-symptomatic testing would need to be introduced into neonatal screening programs. However, amelioration or even stopping the disease progression in patients with SMA type II or III, in whom disease symptoms have already manifested, is also a key aim of research, where clinical translation is not dependent on neonatal screening for maximal effectiveness.

Recent breakthroughs in our understanding of pathways acting downstream from SMN that mediate disease pathogenesis in SMA have greatly expanded the range of potential therapeutic targets for SMA, opening up the possibility of delivering disease-modifying treatments outside of the 'therapeutic time-window' that exists for SMN. Several non-SMN-targeted pathways that contribute to disease pathogenesis in SMA have been reported to modulate the SMA phenotype, including ubiquitination pathways and beta-catenin signaling [17], PTEN signaling [18], RSK2 signaling [19], Rho-kinase pathway [20], ERK/AKT pathways [21], and miR-189/mTOR pathways [22]. Likewise, protective SMA genetic modifiers identified in asymptomatic *SMN1*-deleted individuals, such as PLS3 overexpression, which restores SMN-disturbed actin dynamics, also represent attractive SMN-independent therapeutic targets to stabilize and improve the neuromuscular function [23,24].

Major, achievable, ambitions for the future are therefore: i) to develop drug-based therapies that can extend the therapeutic time-window, in particular by stabilizing the neuromuscular system for a longer period, thereby facilitating a greater therapeutic benefit from parallel delivery of *SMN*-targeted therapies (such as AONs or gene therapy) and/or act to stabilize the neuromuscular system in their own right beyond the time-window that exists for *SMN*-targeted therapies; ii) to identify in SMA cells and animal models new targets acting either independently or downstream from SMN, and to develop and test new AONs/drugs acting on these new targets/pathways; iii) to validate new effective gene therapy treatments for SMA, based on the delivery of scAAV9 vectors designed to overexpress or to silence newfound targets for SMA, acting either independently or downstream from SMN and 4) to include SMA neonatal genetic screening.

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Declaration of interest

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Current Opinion in Genetics & Development

How genetic modifiers influence the phenotype of spinal muscular atrophy and suggest future therapeutic approaches

Brunhilde Wirth, Lutz Garbes and Markus Riessland

Both complex disorders and monogenetic diseases are often modulated in their phenotype by further genetic, epigenetic or extrinsic factors. This gives rise to extensive phenotypic variability and potentially protection from disease manifestations, known as incomplete penetrance. Approaches including whole transcriptome, exome, genome, methylome or proteome analyses of highly discordant phenotypes in a few individuals harboring mutations at the same locus can help to identify these modifiers. This review describes the complexity of modifying factors of one of the most frequent autosomal recessively inherited disorders in humans, spinal muscular atrophy (SMA). We will outline how this knowledge contributes to understanding of the regulatory networks and molecular pathology of SMA and how this knowledge will influence future approaches to therapies.

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Introduction

Identification of modifiers is challenging and, therefore, relatively few genetic modifiers have been detected so far (reviewed in [1–6]; only some diseases have been selected due to a restricted number of references). Modifiers can act in multiple ways on the expression or stability of RNA or proteins: The predominant disease-determining gene (in monogenic or oligogenic disorders) can be modulated by *cis*- and *trans*-acting factors, by epigenetic factors, by proteins belonging to the same pathway or network, by proteins involved in stand-alone pathways but converging on a common final pathway or ending in the same biological function or, finally, by extrinsic non-genetic or environmental factors. In concert with the disease determinant, modifiers give rise to a large phenotypic variability, sometimes conferring full protection to an individual carrying a disease-causing mutation, a phenomenon defined as incomplete penetrance. One of the most impressive 'monogenic' diseases shown to be modulated by a large variety of factors is spinal muscular atrophy (SMA), selected herein to exemplify the role of modifiers and the use of this knowledge gained in developing target-driven therapies.

SMA is a common genetic neuromuscular disorder most often leading to childhood lethality. However, this devastating disease has some remarkable and almost unique features. The identification of the modifying factors influencing the SMA phenotype enlarged our understanding of the pathology, molecular and biochemical mechanisms underlying SMA and, most importantly, allowed the development of therapies.

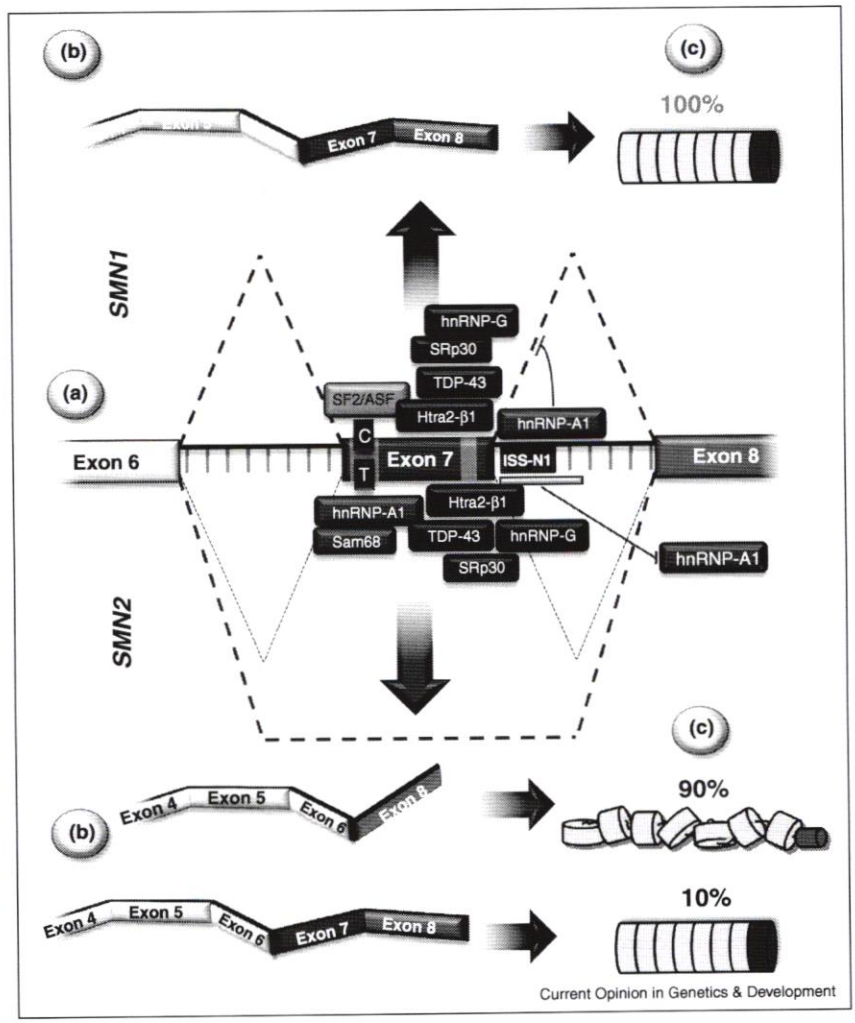
What makes SMA so exceptional and different from other genetic conditions and what do we have to consider for future therapies in order to be successful?

Remarkable features of SMA

First, patients with SMA show a homozygous functional loss of the *survival motor neuron 1* gene (*SMN1*), but all patients carry one or more *SMN2* copies that modulate the severity of the disease [7,8••,9] and can be targeted by small molecules and drugs (reviewed in [10]). Second, about 94% of all SMA patients carry the same type of mutation, which allows simple molecular genetic testing [11]. Third, SMA carriers with frequencies varying between 1:8 and 1:105 among various populations can be easily and reliably identified by use of quantitative PCR or MLPA [7,12,13]. In the European population the frequency of SMA carriers is 1:35 which makes SMA the second most frequent autosomal recessively inherited condition. Fourth, a duplication of about 500 kb on chromosome 5q13.2 including *SMN* occurred late in evolution, in primates, while the development of two different *SMN* genes is human-specific [14]. The location of the *SMN* copies within a CNV makes this region prone to *de novo* deletions, duplications and gene conversions which are found in 2% of SMA patients [15]. The transgenic insertion of the human-specific *SMN2* onto a null Smn background of other species allowed the generation of humanized SMA mouse and pig models, which have been extremely useful in deciphering the pathology of SMA and for development of SMA therapies [16,17••]. Fifth, the main functional difference between *SMN1* and *SMN2* is a single, translationally silent nucleotide exchange in exon 7. It affects an exonic splicing enhancer and thereby impairs correct splicing of *SMN2* so that only very low amounts of full-length (FL) transcripts are produced while the majority of transcripts lack exon 7 [8••,18••]. Sixth, importantly, both FL-*SMN1* and FL-*SMN2* transcripts

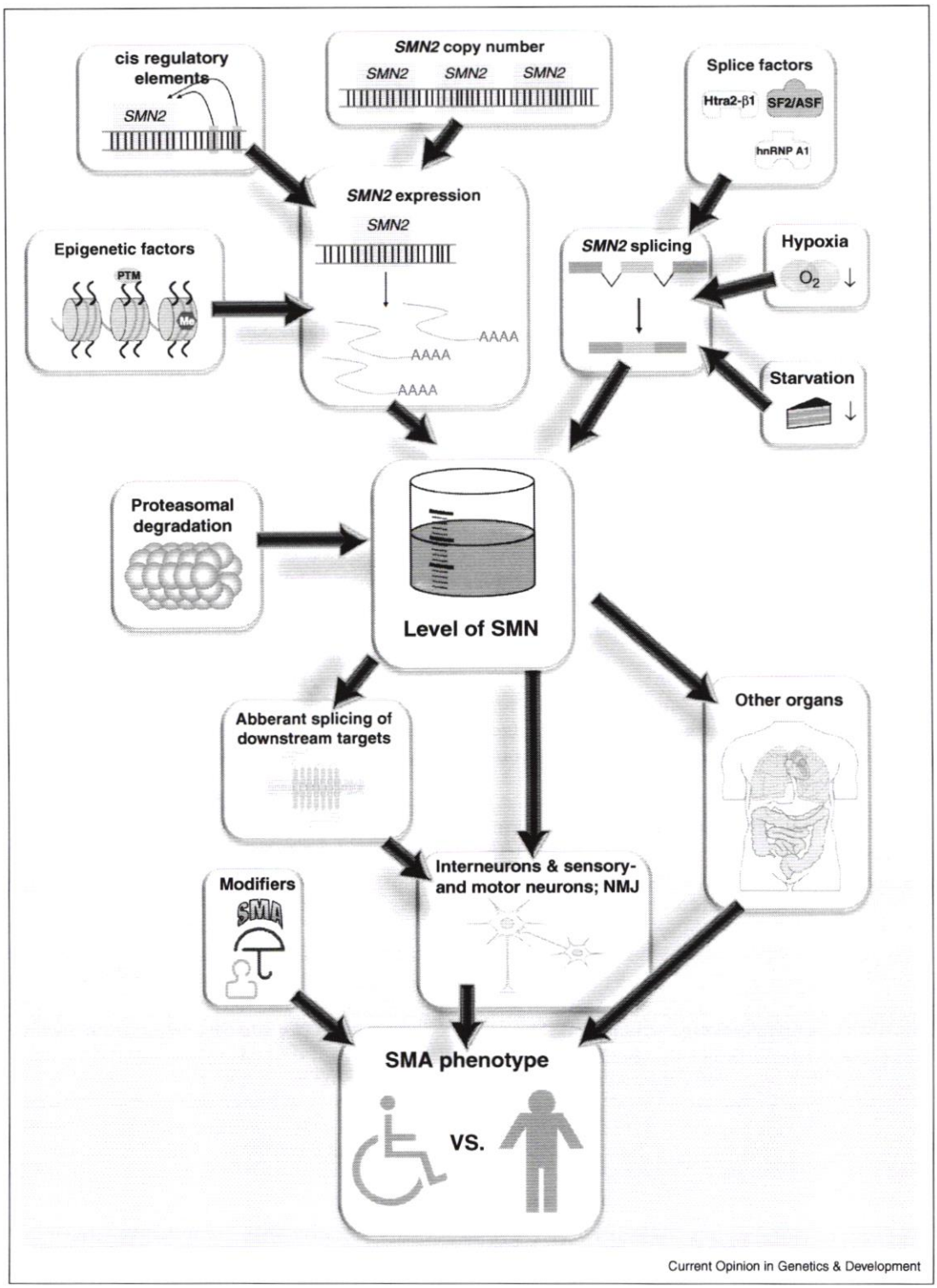
encode an identical SMN protein — a finding that is essential for therapeutic strategies aiming to increase *SMN* expression levels [8••]. Seventh, discordant families with large phenotypic discrepancies pointed towards factors/pathways able to circumvent the detrimental impact of reduced SMN levels on motor neurons. Modifiers such as Plastin 3 (PLS3) have shown to fully protect against SMA in humans carrying a homozygous deletion of *SMN1* [19••]. This knowledge can be further used for the development of SMN-independent therapeutic strategies [20]. Eighth, exogenous factors such as lack of nutrition or hypoxia reduce FL-*SMN2* [21••,22•]. Additional support of SMA patients or SMA mice with oxygen and appropriate nutritional support can partially counteract this negative impact [23,24]. Ninth, despite ubiquitous SMN expression, reduced SMN levels mainly affect motor neurons in the ventral horns of the spinal cord. However, also other neuronal circuits including sensory and interneurons seem

Figure 1



Cis and *trans* regulation of *SMN1* and *SMN2* splicing and its impact on translation. Schematic overview of splicing processes of the *SMN* exon 7 and its consequences on RNA level and protein level. **(a)** Magnification of the exons 6–8 of the *SMN1* and *SMN2* pre-mRNA. The C to T transition at the beginning of exon 7 is highlighted. Both exonic splicing enhancers within exon 7 are shown in gray. Splice factors promoting exon 7 inclusion are depicted in dark gray, while factors favouring exon 7 skipping are marked in red. SF2/ASF, which is displaced by hnRNP-A1 and Sam68 in the *SMN2* splice process, is marked in light gray. Dashed lines indicate the joining of exons during the splicing processes, which is adumbrated by the arrows. Shown in red is the intronic splicing silencer ISS-N1, which is bound by hnRNP-A1. A therapeutic antisense oligo preventing hnRNP-A1 binding is highlighted in yellow. **(b)** Mature mRNA products of both *SMN1* and *SMN2*. While splicing of *SMN1* pre-mRNA produces 100% of full-length transcripts comprising exons 1 to 8 (FL-*SMN1*), *SMN2* produces only about 10% FL-*SMN2*. The remaining 90% of *SMN2* transcripts lack exon 7 (*SMN2Δ7*). Arrows indicate the translation process. **(c)** Final protein products of both *SMN* genes. In contrast to *SMN1*, *SMN2* predominantly produces an unstable truncated protein, which is rapidly degraded.

Figure 2



Schematic overview of the different factors influencing the level of SMN protein and, ultimately, contributing to the severity of the SMA phenotype. The *SMN2* gene copy number directly influences the SMN protein amount, since *SMN2* is the only source of SMN protein in SMA patients. The level of expression of each *SMN2* gene copy can be modified by *cis*-regulatory elements (sequence variations) as well as epigenetic features like *SMN2*

to directly influence the function of motor neurons [25••,26•,27••]. In severe type I SMA patients and SMA mice additional organs such as heart, lung, pancreas, intestine and vascularity are affected turning SMA from a pure motor neuron disorder into a multi-systemic disorder [28,29•,30,31,73]. These findings are essential for future therapeutic considerations. Tenth, postnatal therapies using antisense oligonucleotides facilitating correct splicing or gene replacement therapies using virally transduced *SMN1* rescued the phenotype in SMA mice, however, only when administered in the first three days of life [32••,33••]. This emphasizes the importance of pre-symptomatic treatment and the development of neonatal screening programs. Eleventh, some drugs, such as HDAC inhibitors can only slightly improve the disease outcome but not rescue the SMA phenotype [34,35]. Nevertheless, they might be essential to further maintain *SMN2* expression during a patient's lifetime. Twelfth, SMA can be considered as a developmental and neurodegenerative disorder in severely affected SMA patients and a neurodegenerative disorder in milder affected SMA individuals.

A schematic overview of the *cis* and *trans* regulation of *SMN1* and *SMN2* splicing and its impact on translation is shown in Figure 1 while a schematic overview of the different factors influencing the level of SMN protein and, ultimately, contributing to the severity of the SMA phenotype is shown in Figure 2.

This review will mainly address future requirements for SMA therapies on the basis of our most recent knowledge of SMA modifiers, its pathology and affected tissues in SMA patients and mice.

SMN2, the main modifier of SMA severity and the primary target for therapy

SMN2 copy number and *cis*-regulatory factors

The severity of SMA differs significantly and ranges from the severe form (type I) with early onset in the first six months of life, inability to sit or walk and death before two years of age, to the adult form (type IV) with onset after 30 years of age and only mild motor impairment [36]. While all patients show the same homozygous functional loss of *SMN1* as a consequence of either deletions, gene conversions or, rarely, subtle mutations, they all carry at least one to up to six *SMN2* copies (reviewed in [12,37] and [9]). Each *SMN2* copy produces around 10% FL-*SMN2* transcripts and protein. Consequently, a low *SMN2* copy number is associated with a severe phenotype, whereas a high *SMN2* copy number is associated with a mild phenotype. Hence, most type I patients carry two *SMN2* copies, type II three *SMN2* copies, type III three or four *SMN2* copies and type IV four to six *SMN2* copies [7,9]. Although this inverse correlation is strong, it is not absolute and should not be used for the prediction of the SMA subtype [7]. Furthermore, it has to be taken into consideration that *SMN2* copy number quantification relies on the analysis of the 3′-end of the *SMN2* genes only. Consequently, overseen deletions or duplications at the 5′-end may influence the *SMN2* transcription. *SMN* exon 7 splicing is regulated by exonic and intronic splicing enhancers and silencers (reviewed in [37,38]). Thus, mutations within *SMN2* may abrogate *cis*-regulatory elements leading to an increase in or decrease of FL-*SMN2* levels. For example, c.859G>C disrupts an exonic splicing silencer, leading to an up-regulation of FL-*SMN2* and therefore individuals carrying this variant within their single *SMN2* copy are only mildly affected [39].

Trans-regulatory splicing factors acting on *SMN2*

In addition to *cis*-regulatory elements, a plethora of *trans* splicing factors are involved in *SMN2* exon 7 splicing (reviewed in [37,40]). Overexpression of Htra2-β1 (also known as SFRS10) for example dramatically increases FL-*SMN2 in vitro*. Similar results were achieved with bifunctional oligonucleotide enhancers recruiting SFRS10 [41]. However, the motor neuron-specific knockout of *Sfrs10* in mice had no impact on the phenotype, underlining the complexity and redundancy of the *in vivo* splice regulation, but also the contribution of other neuronal cell types in SMA as recently nicely illustrated by Lotti *et al.* and Imlach *et al.* [25••,27••,42].

Epigenetic modifiers acting on *SMN2*

Moreover, the level of FL-*SMN2* is influenced by epigenetic factors including histone acetylation and methylation of CpG islands within the *SMN2* promoter (reviewed in [37] and [43]). Various histone deacetylase inhibitors (HDACi) such as the short-chain fatty acids valproic acid (VPA), sodium butyrate (NaBu) and phenylbutyrate, as well as the hydroxamic acids LBH589, SAHA, TSA, JNJ-26481585 or the benzamide M344 up-regulate *SMN* RNA and protein levels *in vitro*. Some showed to be successful also in animal studies, leading to improved motor function and 15-30% prolonged survival [44–48,49,50••,73]. Importantly, the effect is

(Figure 2 Legend Continued) promoter DNA methylation (Me) or post-translational modifications (PTM). The second major regulatory mechanism influencing SMN protein levels is *SMN2* splicing, which in turn is regulated by splice factors as well as exogenous factors like hypoxia or starvation. Since SMN is degraded by the ubiquitin proteasome system, its activation or inhibition has also a direct effect on SMN protein level. Reduced SMN amounts lead to the degeneration of motor neurons, interneurons and sensory neurons which will result in weakening and atrophy of muscles, the cardinal symptoms of SMA. SMN deficiency leads to widespread splice defects in U12 snRNP-dependent mRNAs such as Stasimon, which influences the interneuron integrity and finally the motor neuron survival. Besides its impact on nervous circuits, a reduced SMN level also leads to malfunction and dysplasia of other organ systems like the intestine, heart, lung, vascularity, etc. Finally, SMN is influenced by further modifying factors such as plastin 3, an actin bundling protein that influences the F-actin dynamics, a crucial process for axon growth and NMJ function.

strongly associated with the response to HDACi treatment as shown in therapies with VPA in humans. Only about 1/3 of patients are responding positively to VPA and therefore clinical trials may be stratified on the basis of the SMN expression levels in order to show a significant impact [46,51•]. Furthermore, Garbes *et al.* showed that the response to VPA is mostly concordant between blood, fibroblasts and neurons differentiated from induced pluripotent stem cells (iPSCs) of the same patient and that the VPA response is suppressed in *vitro* by an increased expression of CD36, a fatty acid translocase [51•].

Factors influencing the SMN protein stability

SMN is degraded by the ubiquitin proteasomal system as shown in *in vitro* studies and its degradation can be counteracted either directly by proteasome inhibitors MG132 and lactacystin or indirectly by HDACi such as LBH589 [45,52]. Treatment of SMA mice with the proteasome inhibitor bortezomib, which is not blood–brain-barrier permeable, increased the muscle function without extending the lifespan. However, a combined treatment with bortezomib and TSA exceeded the lifespan of TSA treatment alone, emphasizing, first, the importance of CNS drugs that are able to pass the blood–brain barrier, but also second, the additive effect of drugs that activate/stabilize SMN in muscles [53].

External factors acting on *SMN2*

Most strikingly, starvation almost fully abolishes correct *SMN2* splicing as observed in disease end-stage SMA mice, which are extremely weak and severely malnourished [21••]. Indeed, supplementary nutrition has a positive impact on survival in mice [23]. Since SMN is involved in snRNP assembly and splicing, lack of nutrients might down regulate all anabolic processes.

Hypoxia also strongly reduces FL-*SMN2* levels. In line with these observations, respiratory intervention showed a significant improvement in SMA mice and patients [22•,24] (Figure 2).

Both yet identified exogenous factors accelerate disease progression. Possible underlying mechanisms are oxidative stress, proteasomal degradation or increased aberrantly spliced transcripts [21••,54,55], which collectively may cause the rapid death of type I SMA patients or severely affected SMA mice.

Therapies aimed at enhancing SMN levels

Development of SMA therapies has been a focus of researchers for many years and a large number of strategies have been developed to increase or stabilize *SMN2* RNA and protein levels or to reconstitute *SMN1* by gene therapy (recently reviewed in [10,56]).

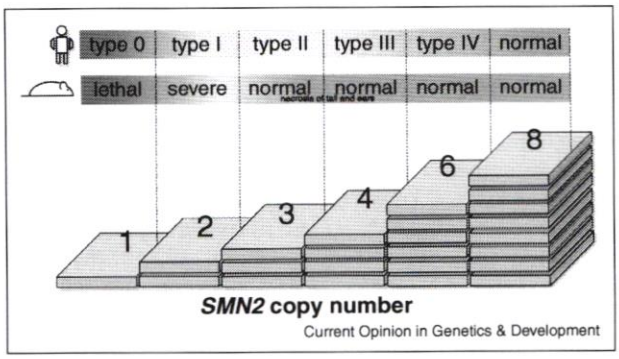
The two most effective therapeutic strategies able to rescue the severe SMA phenotype in mice are: first, increase in FL-*SMN2* levels by the use of antisense oligonucleotides (AON) that block a splice silencer localized in intron 7 [57,58] and secondly, restoration of SMN protein by gene therapy using AAV9 (adeno-associated virus 9) expressing FL-*SMN1* [33••,59]. Both strategies were shown to be highly efficient in mice; however, only when administered in the first three days of life. Administration later in life had little or no effect, highlighting that first, type I SMA is a developmental disease and for a successful therapy SMN reconstitution is required during this crucial period of life and secondly, efficient therapies require pre-symptomatic treatment. The first clinical trial in humans aimed to evaluate the safety of an AON (ISIS-SMNRx) is in progress (www.clinicaltrial.org; NCT01494701). Furthermore, multiple drugs able to directly or indirectly restore the function of NMJs have been developed and are in various pre-clinical or clinical approaches (reviewed in [10,56]).

Plastin 3, a modifier of SMA

In rare cases, siblings with identical *SMN1/SMN2* genotypes reveal discordant phenotypes, from affected to fully asymptomatic, suggesting that modifying factors protect against the development of the disease [19••]. Since these are rare observations, linkage or association studies cannot be applied in most cases. However, it turned out that comparison of whole transcriptomes from only a few affected *versus* unaffected siblings using lymphoblastoid cell lines can be extremely fruitful. Hence, using such an approach *Plastin 3* (*PLS3*) has been identified as the first fully protective modifier of SMA in humans [19••] (Figure 2). PLS3 is an F-actin bundling protein that influences the G/F-actin ratio, which is essential during axonal growth and pathfinding [19,60]. Overexpression of *PLS3* rescues the axonal growth defects observed in zebrafish with reduced *smn* levels [19••,61]. Similar observations were made in cultured motor neurons from SMA mouse embryos [19••]. Ackermann *et al.*, who generated a transgenic mouse overexpressing PLS3 on an SMA background, revealed that all F-actin-associated processes at the level of the NMJs are affected in SMA animals, but were rescued when PLS3 was overexpressed. Most importantly, delayed axon pruning counteracts the poor connectivity observed in SMA NMJs [62••]. Concordantly, SMA mice revealed a significant increase in active RhoA (RhoA-GTP), a major upstream regulator of the actin cytoskeleton [63]. Treatment of SMA mice with fasudil, a Rho A inhibitor, significantly improved the motor abilities and survival [20]. This example nicely illustrates the importance of modifiers for the understanding of the disease pathology and the development of strategies to overcome the main causative gene defect.

Moreover, Next-generation sequencing of whole exomes or genomes will hopefully facilitate the identification of modifiers in the future [64,65].

Figure 3



Different requirement of *SMN2* copies to fully compensate the *SMN1* loss in humans and mice; The correlation of the *SMN2* copy number and the phenotype of both SMA patients and SMA animal models is shown. Since each *SMN2* copy produces about 10% of FL-*SMN2*, the amount of FL-SMN protein increases likewise. The more SMN protein is produced by *SMN2* copies throughout development, the milder is the resulting SMA phenotype. Note the different requirement of FL-*SMN2* levels in humans and mice to fully compensate for the lack of *SMN1* and *Smn*, respectively. In mice a slightly increased level warranted by three instead of two *SMN2* copies leads to an almost normal phenotype. In humans only eight copies lead to a normal phenotype.

What can we learn from the pathology for further therapies in SMA?

Human and mice seem to require different levels of full-length SMN protein (Figure 3). While mice carrying two *SMN2* transgenic copies on murine null *Smn* background exhibit a severe SMA phenotype, three copies are already sufficient for an inconspicuous phenotype. Instead, in human even six copies are not sufficient to protect from an adult-onset SMA. Thus, humans require not only a much larger amount of full-length SMN, but also the dependency between copy number and disease severity is different. The different requirement of SMN between the two species renders a faithful comparison difficult. Generation of large animals like SMA pigs are in progress and may better resemble the human SMA phenotype and may thus be a more appropriate model for pre-clinical studies [66].

Although SMN is ubiquitously expressed and has an important housekeeping function in snRNP biogenesis and splicing, reduced SMN levels predominantly affect the proper function of lower motor neurons [16,17••,67]. However, the pathology varies between species. In fish, reduced Smn levels cause impaired axonal growth, early truncation and branching of motor axons [68]. In mammals, the motor neuron somata and axonal growth are not primarily affected. Instead the neuromuscular junctions show massive functional impairment. SMA NMJs show reduced synaptic connectivity, endplate size, synaptic vesicle release, active zones, docked vesicle at presynaptic membrane, number of mitochondria as well as disturbed Ca^{2+} homeostasis, finally leading to a reduced neurotransmission (reviewed in [69] and [62••]). Recently, it has been reported that also afferent synaptic inputs, sensory circuits and terminal Schwann cells are affected and contribute to the SMA phenotype [25••,26•,27••,70]. These findings suggest that processes and neuronal circuits essential for development and maturation of NMJs (severe SMA form) but also for maintenance of NMJs (mild SMA form) are disturbed in SMA and that targeting all these processes by drugs, small molecules or SMN replacement need to be addressed in a certain timeframe in order to guarantee a life long beneficial effect for the SMA patient.

Additionally, there is increasing evidence that also other organs are impaired in severe SMA mice and type I SMA patients, particularly in those carrying only one *SMN2* copy. In mice recapitulating the human SMA phenotype, these include: a dwarf-like phenotype due to an impaired insulin-like growth factor 1 signalling axis, reduced heart size with septum defects and arrhythmia, defects in vascularization with necrosis of digits, tail and ear pinnae due to poor capillary development, lung emphysema with ruptured alveolar septa, reduced and degenerated villi of the small intestine with massive diarrhea [28–31,32••,71–73]. In agreement with these results, tissue-specific knock-out of *Smn* in any cell type impairs the function of the involved organ and causes lethality as expected on the basis of the important housekeeping function of SMN in snRNP biogenesis and splicing [67,74,75]. Therefore, a systemic replacement of SMN, particularly in type I SMA patients will be fundamental in order to be long term beneficial for the patient.

Conclusion

Homozygous functional impairment of *SMN1* causes SMA in the majority of individuals except for those carrying a protective SMA modifier [8••,19••,76]. In humans, one or two *SMN2* copies are usually associated with type I SMA [37]. In mice, one transgenic *SMN2* copy on a murine null *Smn* background does not protect from early lethality and two copies allow a survival of a few days up to 15 days depending on the genetic background [62••,77]. The major defect in these mice is found at the NMJ level with poor axonal connectivity and disturbed maturation. PLS3, a protective modifier of SMA, rescues the NMJ function by stabilizing F-actin-dependent processes. Most importantly, PLS3 overexpression delays axonal pruning — the retraction of exuberant axons during the first two weeks of life and part of the maturation process of NMJs — thus counteracting the poor pre-synaptic connectivity and facilitating the maturation of NMJs [62••]. We therefore conclude that SMA type I is primarily a neurodevelopmental disease. In support of this view, all pre-clinical trials emphasize that a rescue can be achieved only when SMN is restored in the first three days of life [32••]. Consequently, in order to avoid SMA development in humans, pre-symptomatic

treatment in the first months of life will be mandatory. Therefore, *SMN1* deletion testing needs to be included in neonatal screening programs in the future. In two states of the U.S., neonatal screening for SMA is already applied. Thus, this early diagnosis combined with promising therapeutic approaches on the basis of AONs are 'trend-setting' achievements and will hopefully change the future of *SMN1*-deleted individuals.

In contrast to severe SMA, in milder SMA patients or mice, the NMJs are formed but their maintenance is impaired. This could be due to reduced synaptic vesicle trafficking and dynamics in the pre-synapse [62••,78,79]. We would therefore consider milder SMA as a neurodegenerative disease.

Furthermore, animal models taught us that it is not sufficient to rescue the SMN level in motor neurons, but also other organs might be affected in long living SMA patients and, therefore, a systemic compensation of otherwise insufficient SMN amounts might be mandatory. Starting therapy after symptoms have occurred will at the best slightly improve motor abilities or stop disease progression, but will not cure SMA.

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Spinal Muscular Atrophy: From Gene to Therapy

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The molecular basis of spinal muscular atrophy (SMA), an autosomal recessive neuromuscular disorder, is the homozygous loss of the survival motor neuron gene 1 (*SMN1*). A nearly identical copy of the *SMN1* gene, called *SMN2*, modulates the disease severity. The functional difference between both genes is a translationally silent mutation that, however, disrupts an exonic splicing enhancer causing exon 7 skipping in most *SMN2* transcripts. Only 10% of *SMN2* transcripts encode functional full-length protein identical to SMN1. Transcriptional activation, facilitation of correct *SMN2* splicing, or stabilization of the protein are considered as strategies for SMA therapy. Among various drugs, histone deacetylase inhibitors such as valproic acid (VPA) or 4-phenylbutyrate (PBA) have been shown to increase *SMN2*-derived RNA and protein levels. Recently, in vivo activation of the *SMN* gene was shown in VPA-treated SMA patients and carriers. Clinical trials are underway to investigate the effect of VPA and PBA on motor function in SMA patients.
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KEYWORDS spinal muscular atrophy, survival motor neuron, neuromuscular disorder, valproic acid, SMA therapy

Spinal muscular atrophies (SMAs) are a genetically heterogeneous group of neuromuscular disorders with an autosomal recessive, autosomal dominant, or X-linked recessive mode of inheritance. The majority of patients present autosomal recessive inheritance with proximal manifestation of muscle weakness and atrophy defined as autosomal recessive proximal SMA. With an incidence of about 1:10,000, SMA is one of the most frequent autosomal recessive disorders in humans.[1,2] The carrier frequency, determined by direct molecular genetic testing, is 1:35.[3,4]

The clinical features of the disease are basically caused by the progressive loss of alpha motor neurons in the anterior horns of the spinal cord, which leads to symmetrical weakness and atrophy of the proximal voluntary muscles of legs, arms, and eventually of the entire trunk during disease progression. Electromyographic investigations in patients show a pattern of denervation, typically without sensory involvement or marked decrease in nerve conduction velocity. Histochemical analyses of muscle biopsies provide evidence of skeletal muscle denervation, with groups of atrophic and hypertrophic fibers or fiber-type grouping most often found in chronic cases.

Because the disease severity of SMA is highly variable, the International SMA Consortium defined 4 clinical groups depending on the age of onset and achieved motor abilities.[5,6]

1. Type I SMA (acute form, Werdnig-Hoffmann disease, MIM #253300) is the most severe form with generalized muscle weakness and hypotonia ("floppy infant") and a disease onset within the first 6 months of life. The children are never able to sit or walk and usually die within the first 2 years.
2. Type II SMA (intermediate form, MIM #253550) patients are able to sit but never able to walk unaided, usually present first symptoms after the first 6 months of life, and survive beyond 2 years.
3. Type III SMA (juvenile SMA, Kugelberg-Welander disease, MIM #253400) patients are able to sit and walk, and the lifespan is not reduced. Disease onset before the age of 3 years is classified as type IIIa, whereas an age of onset beyond 3 years is classified as type IIIb SMA. There is a marked difference in the preservation of ambulatory capacity between type IIIa and IIIb. Whereas only 44% of the type IIIa individuals are still able to walk by the age of 20 years, 90% of type IIIb patients of the same age have retained this ability.[6]
4. Type IV SMA (adult form, MIM #271150) patients are comparatively mildly affected with an age of onset later than 30 years; they have a normal life expectancy.

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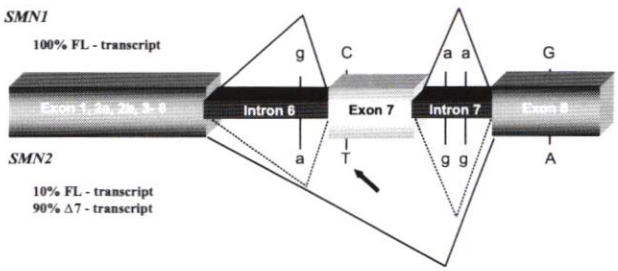

Figure 1 Genomic structure, nucleotide, and splicing differences between *SMN1* and *SMN2*. The *SMN* gene copies can be distinguished by 5 nucleotide exchanges, of which only the C-to-T transition in exon 7 is localized within the coding region. This nucleotide exchange in exon 7 is a translationally silent mutation. Therefore, full-length *SMN1* and full-length *SMN2* mRNA encode identical proteins of 294 amino acids. However, the C-to-T transition disrupts an exonic splicing enhancer resulting in alternative splicing of *SMN2* pre-mRNA and skipping of exon 7. *SMN2*Δ7 transcripts encode a truncated and unstable protein.

Molecular Genetic Basis of SMA: Survival Motor Neuron Gene and Transcripts

Type I, II, and III SMA were mapped by linkage analysis to the chromosomal region 5q11.2-q13.3.[7-9] In 1995, the survival of motor neuron gene 1 (*SMN1*) was identified as the SMA disease-determining gene.[10] The *SMN* gene exists in 2 copies, *SMN1* and *SMN2*, which are localized within a duplicated and inverted chromosomal segment of around 500 kb on 5q13. The 2 *SMN* copies are almost identical except for 5 base pair exchanges that are all localized within the 3′end of the genes (Fig 1).[10,11] However, only the C-to-T transition at position + 6 of exon 7 (c.840C>T) is localized within the coding region. Although it is a silent mutation and therefore not affecting the amino acid sequence of the encoded protein, it severely affects the correct splicing of exon 7. In contrast to *SMN1* which almost exclusively produces correctly spliced full-length (FL) *SMN1* transcripts, *SMN2* produces only ~10% FL-*SMN2* transcripts but 90% of alternatively spliced transcripts that lack exon 7 (*SMN2*Δ7).[10,12]

SMN exon 7 spans 54 bp and is characterized by a weak 3′ splice site.[13] To be recognized by the splicing machinery, additional auxiliary splicing elements are required. Inclusion of exon 7 into *SMN* messenger RNA (mRNA) is regulated by a large number of positive-acting *cis* elements, so-called exonic splicing enhancers (ESEs) or intronic splicing enhancers and by negative-acting *cis* elements termed exonic splicing silencers (ESSs) or intronic splicing silencers (ISSs). These *cis* elements are recognized by *trans*-acting splicing proteins: serine-arginine–rich (SR) proteins or SR-like proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs).

An important ESE is localized at the 5′end of *SMN1* exon 7 and recognized by the splicing factor SF2/ASF (Fig 2). However, this ESE sequence is destroyed by the c.840C>T transition in *SMN2* such that SF2/ASF is not recruited anymore.[14-16] Consequently, exon 7 is not recognized by the splicing machinery, resulting in the preferred generation of alternatively spliced *SMN2*Δ7 transcripts. Additionally, it has been suggested that the C-to-T transition in *SMN2* creates an ESS for hnRNP A1, enhancing the skipping of exon 7.[17] Meanwhile, it was found that hnRNP A1 indeed has a strong inhibitory effect on exon 7 inclusion, but this observation is independent of the C-to-T transition and, therefore, an indirect event not specific to *SMN2*.[14]

In the central part of exon 7, a strongly acting purin-rich ESE is recognized by the SR-like splicing factor Htra2-β1 and a number of further splicing proteins (SRp30c, hnRNP G, and RBM), which all together facilitate the inclusion of exon 7 (Fig 2).[18-20] This interplay between the ESE, Htra2-β1, and the other splicing factors is most likely responsible for the ~10% of correctly spliced FL-*SMN2* transcripts. Overexpression of Htra2-β1 restored the FL-*SMN2* transcript level to almost 80%.[18,19]

Another inhibitory tract (ESS) consisting of 7 nucleotides was found further downstream near the 3′ end of exon 7. However, no splicing factors that bind to this element are described so far.[21,22]

Among several intronic splicing enhancers and silencers in introns 6 and 7, the recently described intronic splicing

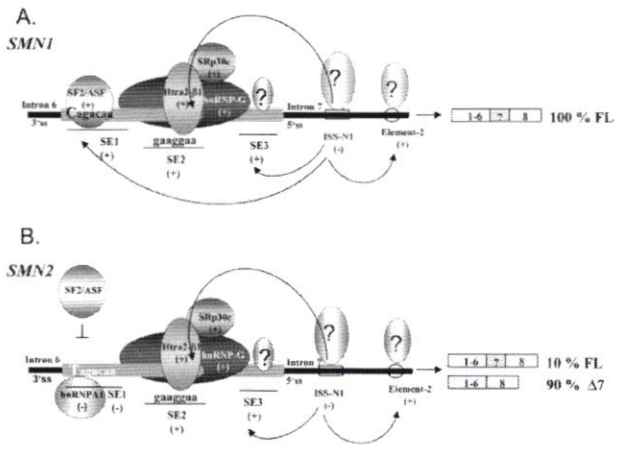

Figure 2 Splicing regulation of *SMN* exon 7. *SMN1* exon 7 contains a heptamer sequence (SE1) at the 5′end that is recognized by the SR-protein SF2/ASF. In *SMN2*, the C-to-T transition disrupts the critical heptamer sequence within SE1, and the splicing factor SF2/ASF cannot bind anymore to exon 7, which results in skipping of this exon.[14,15] Furthermore, the C-to-T transition in *SMN2* exon 7 promotes an inhibitory effect of hnRNP A1 and thus facilitates the exclusion of exon 7.[14,17] Both *SMN1* and *SMN2* contain an ESE in the central part of exon 7 (SE2) that is recognized by Htra2-β1 and its interacting partners hnRNP-G and SRp30c.[18-20] Altogether, they facilitate the inclusion of exon 7 in both *SMN* mRNAs. FL-*SMN1* transcripts are only produced when the SE2 domain is intact.[28] Overexpression of the SE2-dependent splicing factors restores FL-*SMN2* transcript to about 80%.[18,19] At the 3′end of exon 7, another exonic splicing enhancer (SE3) has been identified.[28] Furthermore, intron 7 contains an ISS (ISS-N1) that exerts its function on the positive acting exonic and intronic elements (curved arrows).[25] The *trans*-acting splicing factors that bind the intronic splicing elements or SE3 are not yet identified.

silencer ISS-N1 appears to be the most relevant one.[23-25] ISS-N1 consists of 15 bp, is localized 5 bp downstream of the binding domain for U1 snRNP (uridine-rich small nuclear ribonucleoprotein particle, essential for the splicing process) in intron 7, and exerts a strong effect on the activity of the other positive *cis* elements in exon 7 and intron 7 (Fig 2). The use of sequence-specific antisense oligonucleotides against ISS-N1 resulted in almost complete inclusion of exon 7 into *SMN2* mRNA transcripts, which was shown both in vitro and in cell experiments in vivo.[25]

FL-*SMN* transcripts derived from both *SMN* copies encode an identical FL-SMN protein composed of 294 amino acids, with a stop codon located in exon 7.[10] In comparison, *SMN2*Δ7 transcripts encode a truncated SMN protein of only 282 amino acids. The truncated protein is unstable and shows a reduced oligomerization capacity, which is essential for proper SMN function.[26,28]

The SMN Protein and Its Functions

Both *SMN* genes are ubiquitously expressed. The SMN protein is present in the cytoplasm as well as in the nucleus. In the nucleus, SMN is localized in structures called gemini of coiled bodies (gems), which are often observed in close proximity to or completely overlapping with the Cajal bodies.[29,30] All directly and indirectly interacting SMN partners known so far are listed in Table 1.

SMN is part of a multiprotein complex containing a number of stoichiometrically interacting proteins (referred to as Gemin2-8) and a set of transiently, unstoichiometrically interacting partners, including the Sm proteins, which form part of the spliceosomal U small nuclear ribonucleoproteins (snRNPs).[31-38] It can be assumed from the various direct and indirect interactions with other proteins that have been identified so far, that the SMN protein is involved in a large number of different pathways.

Its major housekeeping function is the biogenesis of snRNPs.[31,32] The U snRNAs are transcribed in the nucleus and subsequently transported to the cytoplasm where they assemble with Sm proteins to form the U snRNP particles. After modification of the m^7 G-cap into an m_3 G-cap, the mature particles are transported back to the nucleus.[33] To catalyze this process, newly translated Sm proteins are bound in a first step to pICln, a component of the PRMT5 complex, which carries out a symmetrical dimethylation of a subset of Sm proteins. In a second step, the methylated Sm proteins are transferred onto the SMN complex, which finally promotes the transfer of the Sm proteins onto the U snRNAs.[34-36]

Thus, the SMN complex and the PRMT5 complex assist the formation of spliceosomal U snRNPs whose major components are the Sm core (formed of 7 Sm proteins: B/B', D1, D2, D3, E, F, and G), 1 (U1, U2 U5) or 2 (U4/U6) uridine-rich RNAs, and several other proteins.[33,37] Functional studies indicated that the SMN complex mediates the formation of spliceosomal U snRNPs in an adenosine triphosphate–dependent manner and thus functions as an RNP chaperone.[29,38] Furthermore, the SMN complex also assembles U7 snRNP, which is required for 3'-end processing of histone pre-mRNAs rather than pre-mRNA splicing and whose Sm core contains a unique combination of Sm and Lsm proteins.[39] Additionally, SMN seems to be involved in pre-mRNA splicing, regulation of transcription, and gene expression.[40-43]

Despite the vast knowledge concerning the SMN biochemistry, it remains unclear how an impairment of snRNP biogenesis, which is a ubiquitous SMN function in all cell types, specifically causes alpha motor neuron degeneration. This raises the question whether SMN fulfils an additional function restricted to alpha motor neurons. Interestingly, immunocytochemical studies have localized SMN in dendrites and axons and suggest a role in the transport of RNA along the axons.[44,45] Zhang and colleagues[46] showed that the SMN protein is localized in granules present in neurites and growth cones of cultured neuronal cells. SMN-containing granules exhibited rapid, bidirectional movements depending on both microtubules and microfilaments.[46] A major finding that supports the idea of a neuron-specific role of SMN in axonal mRNA transport is its interaction with hnRNP R, a protein that binds to the 3'untranslated region of β-actin mRNA, as well as the identification of reduced levels of β-actin mRNA at distal axons and growth cones in motor neurons isolated from SMA-like transgenic mice.[47]

SMN is highly expressed during embryogenesis, but levels decline rapidly after birth. In SMA-transgenic mice, the length of dendrites is significantly reduced, whereas the number of motor neurons is not significantly affected as compared with controls.[47] Conditional neuronal knockout mice (SmnΔ7) lack axonal sprouting.[48] Consistent with these findings in mice, knockdown of the Smn protein by antisense morpholinos in zebrafish embryos has revealed a significant axonal dysmorphology. The axons fail to reach motor neuron endplates because of early branching and truncation, which also strongly suggests an important role of SMN in the pathfinding of axons.[49]

SMN1 Gene Mutations in SMA Patients

Among the 5q13-linked SMA patients, 96% of type I-III SMA patients show homozygous absence of *SMN1* exon 7 and 8 or exon 7 only because of deletions of *SMN1* or gene conversion of *SMN1* into *SMN2* (Fig 3).[10,50-53] In contrast, homozygous absence of *SMN1* is only very rarely found in type IV SMA patients.[54-56]

Based on this relatively uniform mutational spectrum found in type I-III SMA patients, a fast and reliable molecular genetic polymerase chain reaction–based testing is available.[57-59] Among SMA patients with homozygous deletions of *SMN1*, about 90% reveal homozygous absence of both exons 7 and 8, whereas about 10% show only homozygous absence of exon 7 but not of exon 8.[60] The molecular basis for the latter phenomenon is gene conversion, a common mutational mechanism in the SMA region that causes either conversion of *SMN1* into *SMN2* or vice versa.[10,61,62] Gene conversion has been described as a de novo event in rare cases.[58,63,64] The region of conversion can encompass the complete *SMN* gene as well as only a part of it.[61,65]

Besides the homozygous absence of *SMN1*, a minority of

Table 1 The SMN Protein Is Present as Part of a Large Macromolecular Complex Containing a Number of Common Core Components and a Set of Transiently or Substoichiometrically Interacting Partners

SMN complex component	Direct (+)/Indirect (−) SMN Interaction	Function	References
Core components			
Gemin1 (SMN)	+		26,119
Gemin2 (SIP 1)	+	snRNP biogenesis and pre-mRNA splicing	119
Gemin3 (DP103)	+	snRNP biogenesis and pre-mRNA splicing	43,120
Gemin4 (GIP1)	−	snRNP biogenesis and pre-mRNA splicing	121,122
Gemin5 (p175)	+	snRNP biogenesis and pre-mRNA splicing	123
Gemin6	−	snRNP biogenesis and pre-mRNA splicing	124
Gemin7	+	snRNP biogenesis and pre-mRNA splicing	125
Gemin8	−	snRNP biogenesis and pre-mRNA splicing	126
unrip	−	snRNP biogenesis and pre-mRNA splicing	38
Substrates and substoichiometric components			
Sm proteins	+	snRNP biogenesis and pre-mRNA splicing	119,127
LSm4	+	snRNP biogenesis and pre-mRNA splicing	127,128
Fibrillarin	+	Assembly of snoRNPs	129,130
GAR1	+	Assembly of snoRNPs	130
Coilin	+	Recruitment of SMN to Cajal bodies	131
U1-A, U2-A′	Unknown	snRNP biogenesis	119
Profilin	+	Control of actin dynamics	132
ZPR1 (zinc-finger protein 1)	+	Caspase activation and apoptosis; snRNP assembly/maturation	133,134
OSF (osteoclast-stimulating factor)	+	Regulation of osteoclast formation and activity	135
Nucleolin and B23	−	Cell growth and proliferation control, programmed cell death, cell surface signal transduction, and differentiation and maintenance of neural tissues	136
RNA helicase A	+	Transcription	40
RNA polymerase II	−	Transcription	40
hnRNP Q and R	+	RNA transport along the axons	137,138
hsp70 (heat shock protein 70)	Unknown	Posttranslational protein transport	38
Snurportin and importin β	− and +	Transport of snRNPs to nucleus	139
Galectin 1 and 3	−	snRNP biogenesis and pre-mRNA splicing	140
p53	+	Apoptosis	141
ISG20	Unknown	Degradation of single-stranded RNA	142
FGF-2 (fibroblast growth factor 2)	+	Neurotrophic factor for motor neurons	143
mSin3A	Unknown	Transcriptional regulation	144
EWS (Ewing Sarcoma)	+	Transcriptional regulation	145
Bcl-2	+	Antiapoptosis	146
FUSE-binding protein	+	Regulator of transcription and mRNA stability	147
PPP4 (protein phosphatase 4)	−	Ubiquitous protein phosphatase that dephosphorylates serine and threonine residues	148
TGS1 (trimethylguanosine synthase 1)	+	snRNA cap hypermethylase	149
Rpp20	+	Maturation of tRNA and rRNA in RNase P and RNase MRP complexes	150
Viral proteins			
Papilloma virus E2	+	Nuclear transcription activation	41
Minute virus NS1 and NS2	Unknown	Viral replication and a potent transcriptional activator	151,152
Epstein-Barr virus nuclear antigen	Unknown	Transcriptional regulation	153

5q13-linked SMA patients (~4%) exhibit intragenic *SMN1* mutations. Typically, these patients are compound heterozygous with a deletion on one and a subtle mutation on the other chromosome 5.[58,60,65,67,72] About 40 subtle mutations have been identified so far, many of them being missense mutations that disturb the proper SMN1 protein func-

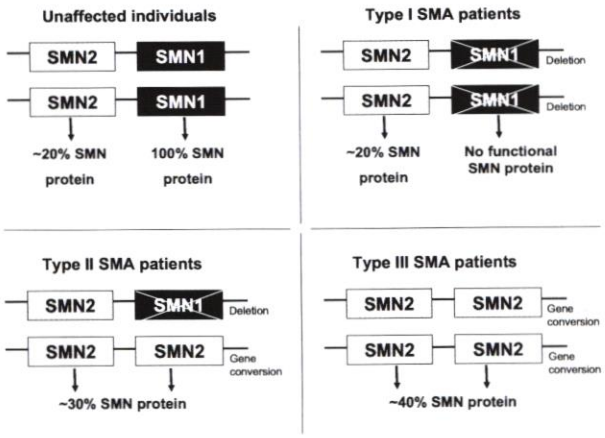

Figure 3 The *SMN1* and *SMN2* genotype usually found in unaffected individuals and type I-III SMA patients. Note that an increased *SMN2* copy number, produced by gene conversion, correlates with a milder SMA phenotype and larger amounts of FL-SMN protein.

tion.[26,66,67] About 3% of all patients who are clinically diagnosed with a phenotype indistinguishable from 5q13-linked proximal SMA fail to show any mutation within the *SMN1* gene, pointing toward genetic heterogeneity.[58]

Homozygous absence of *SMN2*, a genotype found in about 3% to 5% of control individuals, has no apparent phenotypical consequences.[10] The presence of at least 1 fully functional *SMN1* gene is sufficient to protect from SMA. However, there are few reports in which associations between either homozygous deletion of *SMN2* or abnormal *SMN1* copy number and amyotrophic lateral sclerosis or lower motor neuron diseases were reported.[68-70]

Influence of *SMN2* Copy Number on the SMA Phenotype

The major factor influencing the severity of the SMA phenotype is the number of *SMN2* copies,[3,55,71,72] which usually varies between 1 and 4 and rarely reaches up to 8 copies. The underlying mechanism generating an increased number of *SMN2* copies and a reduction or absence of *SMN1* is gene conversion (Fig 3).[61,62,71] Because each *SMN2* copy produces about 10% of FL-*SMN2* transcripts, an increased number of *SMN2* genes is beneficial for SMA patients and thus influences the severity of SMA.[12,71]

The majority of type I SMA patients carry 2 *SMN2* copies, type II SMA patients 3 *SMN2* copies, type IIIa SMA patients (age of onset before 3 years) 3 *SMN2* copies, type IIIb SMA patients (age of onset after 3 years) 4 *SMN2* copies, and type IV 4 to 6 *SMN2* copies.[3,55] Analysis of FL-*SMN2* versus *SMN2*Δ7 transcripts of type I-III SMA patients showed a ratio of about 20: 80 in type I, 30: 70 in type II, and 40: 60 in type III SMA patients.[12] On protein level, substantial differences are found especially when comparing type I and II SMA patients with type III patients and controls.[12,73,74] Individuals carrying 5 or 6 *SMN2* copies develop very mild SMA symptoms (type IV SMA),[55] whereas 8 *SMN2* copies fully protect from developing SMA.[75] Similar phenotypical differences have been observed in transgenic SMA mice carrying 2 to 8 copies of the human *SMN2* gene on a *Smn*-knockout background.[76,77]

Overproduction of *SMN2*Δ7 in double-transgenic SMA mice with the genotype $Smn^{-/-}$;$SMN2^{+}$;*SMN2*Δ7 revealed an increased life span compared with transgenic mice with the genotype $Smn^{-/-}$;$SMN2^{+}$.[78]

Evidence for Further Genes Modifying the Disease Severity

In rare cases, siblings with identical *SMN1* mutations, identical *SMN2* copy numbers, and identical 5q13 haplotypes reveal marked phenotypical discrepancies, reaching from affected to SMA-unaffected, suggesting the existence of modifying genes not linked to chromosome 5q13.[52,79-82] In this context, the gender is of particular interest; except for a few cases, most unaffected but *SMN1*-deleted persons are females. Furthermore, there is a marked deviation from the expected 25% segregation ratio observed among females in type III SMA families. Helmken and colleagues[12] have been able to show that the modifying factor is regulating either the translation rate or the stability of the SMN protein because unaffected subjects with homozygous absence of *SMN1* reveal substantially more SMN protein compared with their affected siblings, whereas the *SMN2* transcript levels were, except for 1 sibpair, similar.[12] This phenomenon was tissue specific and has been shown only in lymphoblastoid cell lines, not in primary fibroblasts derived from phenotypically discordant SMA siblings. In addition to the marked discrepancy concerning the SMN protein level, similar differences were found for SMN1-interacting proteins (Gemin 2, Gemin 3, ZPR1, and hnRNP-G) and for Htra2-β1. Especially the correlation between SMN and Htra2β-1 is highly interesting because Htra2-β1 is involved in pre-mRNA processing of SMN exon 7. Unfortunately, the feedback mechanism by which SMN controls the amount of Htra2-β1 is yet unknown. SMN and Htra2-β1 do not directly interact as shown by coimmunoprecipitation analysis.[12] The identification of the modifying gene could open a new therapeutic strategy to change the course of SMA because that biological pathway would point out a possibility for preventing SMA in case of homozygous absence of *SMN1*.

Heterozygosity Testing for SMA

Based on the fact that SMA affects 1 in 10,000 newborns[1,83] and according to the Hardy-Weinberg equilibrium, the carrier frequency in the general population should be 1:50 individuals. However, this estimation is based on statistical calculation and assumes a lower carrier frequency than that determined by molecular genetic analysis. Direct quantification of *SMN1* copies revealed a heterozygosity frequency of 1:35 in the Caucasian population.[3,4] The carrier frequency even might be slightly higher because carriers with subtle mutations (1.7%) or with 2 *SMN1* copies on 1 chromosome (2.4%) are not detected by this test.[3]

The discrepancy between statistical and molecular genetic studies is most likely the result of the following factors: (1)

some individuals with homozygous absence of *SMN1* are not detected because they are asymptomatic, (2) embryonic lethality of subjects with homozygous large chromosomal deletions including both *SMN* copies, and (3) some very severely affected patients might not have been included into epidemiologic studies.

Quantification of *SMN1* copies is frequently requested in the context of genetic counseling, either to check for an SMA carrier status or as a means to identify patients with compound mutations (deletions and intragenic *SMN1* mutation). Nowadays, a large number of various methods are available for reliable testing.[3,72,84]

Unfortunately, the presence of 2 *SMN1* copies per chromosome (2.4%) or intragenic mutations (1.7%) in some of the detectable *SMN1* genes slightly diminish the sensitivity of the test.[3,58,59,72] Consequently, 4.1% of individuals would be misinterpreted as noncarriers on the basis of the direct *SMN1* determination. The sensitivity of the test in the general population is 95.9%.

Prenatal Diagnosis and Preimplantation Diagnosis for SMA

In families at risk to have a child with SMA, prenatal diagnosis based on chorionic villi samples taken between the 10th and 12th week of gestation or based on an amniotic fluid sample taken between the 14th and 16th week of gestation can be offered. A simple screening that tests for *SMN1* deletions combined with haplotype analysis using polymorphic markers offers almost 100% sensitivity concerning the future risk for the fetus to develop SMA.

In approximately 2% of SMA patients, the *SMN1* deletion is caused by a de novo event that results either from unequal crossing over or from gene conversion, which is mainly of paternal origin. The recurrence risk in these families is similar to that of the general population.[64] However, because germline mosaicism cannot be completely excluded, one should still offer a prenatal diagnosis to these families.[84] Furthermore, preimplantation diagnosis for SMA has been developed and is offered in several countries.[85-90]

Therapeutic Prospects for SMA

Development of a therapy for spinal muscular atrophy is an exceptional challenge for the scientific community. Meanwhile, SMA may become one of the first inherited diseases in humans that may be treated effectively by transcriptional activation and correction of the splicing of a copy gene. Disclosure of the molecular cause of SMA and the molecular basis of the alternative splicing of *SMN2* exon 7 gives rise to the opportunity to develop therapeutic approaches to modulate splicing, transcription, and/or translation regulation of *SMN2*. Various strategies have been considered so far:

(a) Elevation of endogenous FL-SMN protein levels generated by *SMN2*
- Transcriptional *SMN2* activation via the gene promotor
  - Histone deacetylase (HDAC) inhibitors: sodium butyrate, valproic acid, 4-phenylbutyrate, SAHA, M344[91-96]
  - Compounds that regulate DNA methylation of the *SMN2* gene promoter: valproic acid, 5-Aza-2′deoxicytidine[97,98]
  - Other substances: interferon, indoprofen, hydroxyurea[99-101]
- Restoration of the correct splicing of *SMN2* pre-mRNA
  - HDAC inhibitors: sodium butyrate, valproic acid, SAHA, M344[91-96,102]
  - Small antisense RNA molecules[13,25,103,104]
  - Other substances: aclarubicin, sodium vanadate[105,106]
- Translational activation and stabilization of the FL-SMN protein
  - Phosphatases and kinases[107]
  - SMA modifying factors[12]
- Suppression of the *SMN2* stop codon to elongate the SMN2Δ7 protein and improve its stability
  - Aminoglycosides[108]

(b) Compensation of the lack of sufficient SMN protein by:
- Stem cell therapy[109]
- Gene therapy[110-112]

(c) Improvement of motor neuron viability through alternative pathways
- Neurotrophic factors: Cardiotrophin-1[114]
- Neuroprotective compounds: Riluzol[113]
- Regular exercise[115]

The discovery of chemical substances that indeed have the potential ability to implement one of the strategies mentioned earlier seems promising. However, most of the compounds identified so far are not suitable for long-term SMA therapy because of their unfavorable pharmacologic properties including toxicity and other undesirable side effects.

In contrast, some HDAC inhibitors such as valproic acid (VPA) and 4-phenylbutyrate are already approved by the Food and Drug Administration for application in the therapy of various diseases. VPA has successfully been used in long-term therapy of epilepsy as well as for the treatment of mood disorders and migraine.[116] The drug has clinically well-

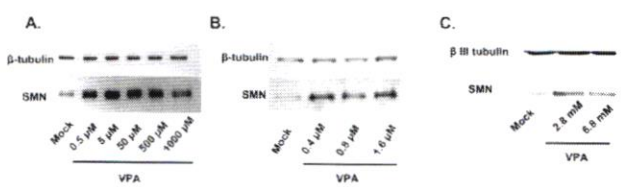

Figure 4 VPA markedly increases SMN protein levels in (A) SMA patient-derived fibroblast cell lines (*SMN1* deleted) after treatment with micromolar VPA-concentrations,[92] (B) human hippocampal brain slice cultures (derived from epilepsy surgery) after treatment with VPA,[95] and (C) motor neuron enriched cell cultures (isolated from rat embryos) treated with millimolar doses of VPA.[95]

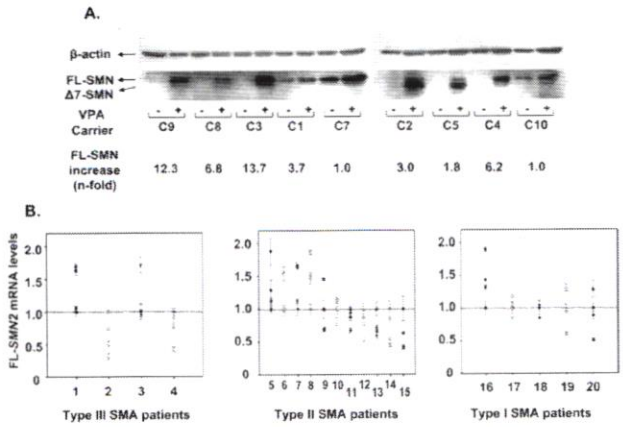

Figure 5 VPA activates the transcription of *SMN* in SMA carriers and SMA patients. In the diagrams, the scale on the y-axis is given in arbitrary units and describes the levels of FL-*SMN2* transcript before and during experimental VPA treatment. (A) Proteins were extracted from mononucleated white blood cells isolated from whole-blood samples obtained before and during VPA treatment of SMA carriers. A substantial increase for FL-SMN protein was observed in most SMA carriers.[117] (B) FL-*SMN2* transcript levels were determined by real-time polymerase chain reaction in 5 blood samples (2 samples taken before treatment and 3 after a VPA serum level of ~70-80 μg/mL was achieved) in 20 SMA patients. Note that 7 patients (1, 3, 5-8, and 16) revealed increased FL-*SMN2* levels, 6 patients (9, 10, 17-20) unchanged, and 7 patients (2, 4, 11-15) decreased FL-*SMN2* transcript levels under VPA treatment.[117]

known and desirable pharmacologic properties including a suitable terminal half-life of 9-18 hours in human serum. In fibroblast cells derived from SMA patients, it has been shown that drug concentrations in therapeutic ranges achieve 2- to 4-fold elevated *SMN2* mRNA and protein levels by increasing gene transcription and driving exon 7 inclusion (Fig 4A).[92] Furthermore, VPA induces *SMN* gene expression in neuronal tissue, the target for a potential SMA therapy; this has been shown in hippocampal brain slice cultures from rat and human (obtained after surgery of epilepsy patients) and in cultured motor neurons from rat embryos (Figs 4B and C).[95] In addition, VPA selectively induces proteasomal degradation of HDAC2, the major enzyme involved in transcriptional *SMN2* gene repression, thus boosting the action on the target gene *SMN2*.

What Can We Learn From First Pilot Trials With VPA and 4-Phenylbutyrate in SMA Carriers and Patients?

A first pilot trial with VPA in 10 parents of SMA patients, each of them carrying 1 *SMN1* and 1 to 3 *SMN2* copies, has been performed for a period of 4 months (VPA serum levels ~80 μg/mL). In 7 of the 10 SMA parents, an increase of FL-*SMN* mRNA levels by 60% to 240% was determined in blood. Particularly substantial was the increase of SMN protein levels in 7/9 carriers, reaching up to ~14-fold higher values under VPA treatment compared with untreated samples (Fig 5A).[117]

Individual experimental curative approaches with VPA in 20 patients with type I, II, and III SMA (VPA serum level ~70-80 μg/mL) revealed elevated FL-*SMN2* mRNA levels in blood from 7 patients and unchanged or even decreased levels in another 13 patients (Fig 5B).[117] An improvement of the clinical picture after 5 to 6 months of treatment was experienced in about 50% of the patients (Wirth, unpublished data, 2006). Some of the patients treated with VPA presented decreased L-carnitin levels that were compensated by substitution with acetyl-carnitin (50 mg/kg body weight/d). The observation of patients with unchanged/decreased FL-*SMN2* transcript levels underscores the need for a biomarker to discriminate between responders and nonresponders to the drug. So far, we do not know if *SMN* expression in blood reflects *SMN* expression in alpha motor neurons and correlates with muscle strength. Therefore, long-term clinical trials in SMA patients that correlate *SMN* expression in blood with individual motor function tests are required. There are 2 large trials with VPA/L-carnitin: one is in progress in type II and III SMA patients in the United States (www.FSMA.org) and one is planned in type I SMA patients in Germany (www.initiative-sma.de).

A pilot trial with phenylbutyrate in SMA patients already revealed promising results.[118] However, only phase III placebo-controlled clinical trials will provide the final proof for a benefit of HDAC inhibitors in SMA therapy.

Neonatal Screening for SMA

Although neonatal screening for SMA is not yet justified, this may rapidly change as soon as clinical trials will prove that certain drugs may be beneficial for SMA patients. The uniform mutation spectrum in SMA facilitates a rapid and reliable genetic test. However, although most SMA patients carry homozygous absence of *SMN1* and will be detected, approximately 3% to 4% of SMA patients carry subtle *SMN1* mutations and will not be identified by the test. On the other hand, some few individuals who will reveal homozygous absence of *SMN1* would never develop SMA because they are protected by an SMA-modifying factor.[12]

Furthermore, although the correlation between SMA phenotype and *SMN2* copies is not absolute, Feldkotter and colleagues[3] were able to calculate the probability of developing a type I, II, or III SMA in a child with homozygous absence of *SMN1* by measuring the *SMN2* copy number. Thus, a child with 1 *SMN2* copy has a risk of >99% and with 2 *SMN2* copies a risk of 97% to develop a type I SMA. A child with 3 *SMN2* copies has a risk of 82.8% to develop a type II SMA, and a child with 4 copies has a risk of 83.6% to develop a type III SMA.[3]

Based on these data, the SMA type can be predicted, which may be useful for selecting the right time point and dose for a potential drug treatment. Neonatal screens will be crucial for a successful SMA therapy. Children at risk to develop SMA should be recognized as soon as possible before first symptoms occur. However, as long as we do not have a clear answer whether any drug is sufficiently beneficial to SMA patients, a neonatal screening for SMA should not be offered.

Conclusions and Perspectives

SMA may become one of the first human-inherited disorders for which our increasing knowledge about the molecular pathogenesis and the regulation of transcription and splicing of the *SMN2* copy gene smoothens the way toward a causal therapy. The features that make SMA a unique therapeutic disease model are as follows: (1) approximately 94% of all patients with SMA show the same mutation, which can be easily identified by simple molecular genetic testing; (2) although *SMN1* is homozygously deleted in patients with SMA, a copy gene (*SMN2*) is present in all patients producing minor amounts of a protein identical to SMN1; and (3) several drugs have the potential to significantly elevate the FL-*SMN2* mRNA/protein level as shown in cell systems as well as in pilot trials in humans. However, only some SMA patients seemed to be positive responders, whereas others were negative responders or failed to show a clear upregulation of full-length *SMN2* transcripts in blood. These results emphasize the need for large clinical trials in which a potential biomarker in blood will be correlated with clinically established outcome measures (ie, motor function tests) and which carefully investigate the effect of drugs on the muscle strength in SMA patients.

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BUDUĆNOST ONKOLOGIJE U HRVATSKOJ

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MULTIDISCIPLINARNI PRISTUP OSOBI S NASLJEDNOM SKLONOŠĆU RAKU DOJKE I JAJNIKA

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Zbog sve veće složenosti liječenja oboljelih od zloćudnih bolesti jedna specijalnost ne može obuhvatiti sve aspekte i oblike dijagnostike i terapije onkološkog bolesnika te je multidisciplinarni bolnički tim preduvjet učinkovitijeg i kvalitetnijeg liječenja. Osim uobičajenih članova tima (obiteljski liječnik, internist-onkolog, radiolog, ginekolog , kirurg, patolog, posebno educirana medicinska sestra i klinički psiholog) čini mi se važnim uključiti molekularnog biologa i kliničkog genetičara. Ovisno o indikaciji, u većini slučejeva će biti konzultirani samo neki članovi tima a tim bi trebao postojati u okviru tercijarnog centra.

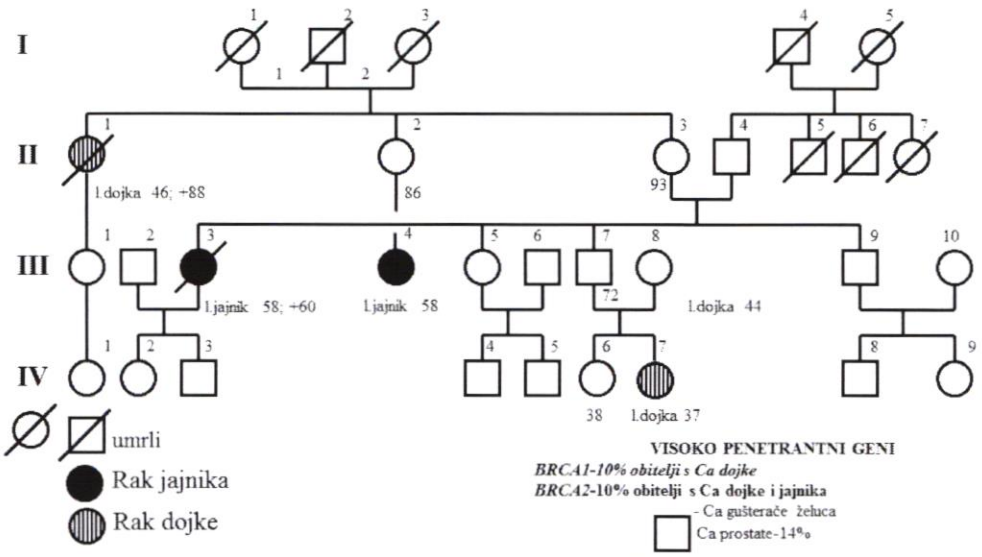
Ako bolesnik ili zdrava osoba ima više srodnika s rakom dojke, jajnika ili neku drugu srodnu vrstu raka u obitelji, potrebno je misliti na genetski uzrok. Takve osobe mogu zahtijevati drugačiju skrb od osoba koje u obitelji nemaju takve vrste raka. Naime, postoje razlike za rizik nastanka tumora na drugoj strani tijela. Isto tako, zdravi članovi obitelji maju veći rizik za pojavu iste bolesti.

Osnovni postulat u pristupu je stratificiranost od primarne do tercijarne službe.

Kako je područje kojim se bavim, klinička genetika, u svom izlaganju ću se osvrnuti više na tu problematiku. Nedostatak kvalificiranih medicinskih genetičara, posebno onkogenetičara postoji svugdje u svijetu, pa tako i kod nas. Ukazala bih na dvije činjenice koje bi trebalo što prije riješiti: Manjak educiranog kadra i nepostojanje nacionalnog programa specijalizacije iz medicinske genetike. Kako potrebe rastu, a rješavanje problema predstavlja proces od više godina, bilo bi korisno što prije integrirati osnove kliničke genetike i genetske specifičnosti određene patologije u sve kliničke specijalizacije i obnavljati znanje organiziranjem tečajeva trajne edukacije . Pokušat ću prikazati potrebu za tim znanjem na primjeru obiteljske sklonosti raku dojke i jajnika (Sl.1). Postavljena su tri pitanja i pet sugeriranih odgovora o kojima će se raspravlati:

1. Koji je gen najvjerojatniji uzročnik raka u obitelji na Sl.1 ?
   a. RET, MEN1
   b. TP53, PTEN
   c. BRCA1, BRCA2
   d. MLH1, MSH2
   e. BRC, ABL

Sl.1



VISOKO PENETRANTNI GENI

BRCA1-10% obitelji s Ca dojke

BRCA2-10% obitelji s Ca dojke i jajnika

- Ca gušterače želuca

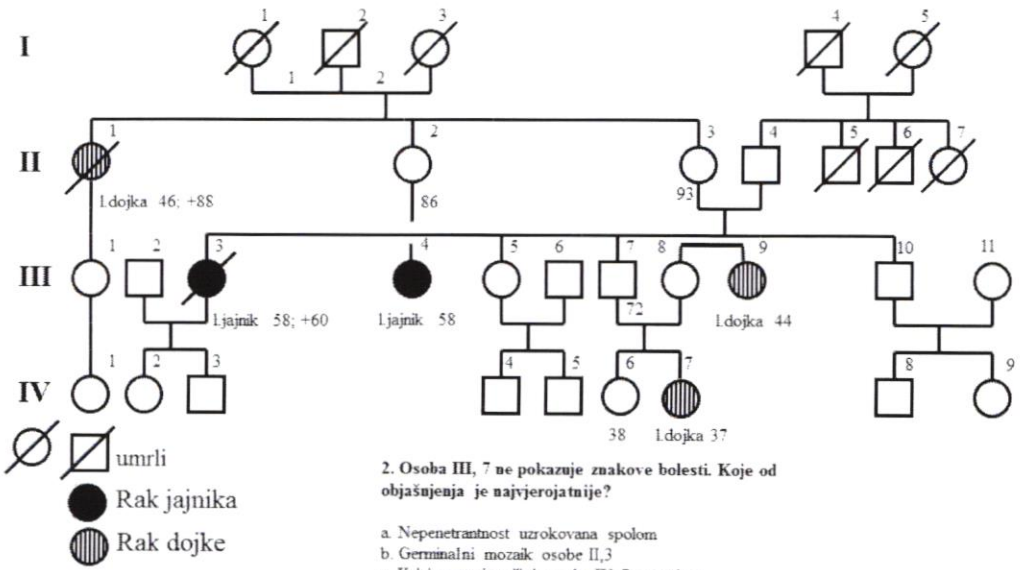
Ca prostate-14%

TP53- RIJETKE germinalne mutacije- Li-Fraumeni sy.-cave RTG

ČESTE somatske mutacije u solidnim tumorima djece

Ca dojke do 30 god. (BRCA1 germ.+BRCA2)

Sl.1a



2. Osoba III, 7 ne pokazuje znakove bolesti. Koje od objašnjenja je najvjerojatnije?

a. Nepenetrantnost uzrokovana spolom
b. Germinalni mozaik osobe II,3
c. Krivi paternitet ili je osoba IV, 7 posvojena
d. Imprinting majke u osoba II,2 i II, 3
e. Spriječen prijenos jer su roditelji klinički zdravi

2. Osoba III,7 (Sl.1) ne pokazuje znakove bolesti a ima bolesnu kćer IV, 7. Koje od objašnjenja je najvjerojatnije?
   a. Nepenetrantnost uzrokovana spolom
   b. Germinalni mozaik osobe II,3
   c. Krivi paternitet ili je osoba IV, 7 posvojena
   d. Imprinting majke u osoba II,2 i II, 3
   e. Spriječen prijenos jer su roditelji klinički zdravi
3. Sl. 1a, Osoba IV,6 (Sl.1a) je zabrinuta zbog rizika da oboli od raka dojke i jajnika i želi se testirati. Liječnik uzima obiteljsku anamnezu i saznaje da osim već poznatih podataka sa strane oca, majka ima sestru kojoj je u 44 . god. dijagnosticiran rak dojke. Koja osoba u obitelji je najprimjerenija da se prva testira?
   a. IV, 6
   b. IV, 7
   c. III, 7
   d. III, 7
   e. II, 3

Osim analize obitelji, ističe se razlika germinalne i somatske mutacije. Germinalne mutacije koje kontroliraju stanični ciklus i mehanizme koji popravljaju DNK imaju za posljedicu nastanak nasljednih sindroma raka. Podaci pokazuju, da germinalne (nasljedne) mutacije uzrokuju rak u približno 5-10% slučajeva, dok većinu slučajeva predstavljaju somatske (ne nasljedne) mutacije. Kliničari trebaju razlikovati te dvije grupe kako bi članovima obitelji s nasljednom sklonošću raku mogli dati presimptomatsku procjenu rizika. Ta procjena može rezultirati savjetima koji smanjuju rizik ili omogućuju rano otkrivanje, bilo da se radi o promjenama u životnim navikama (smanjenje tjelesne težine, prestanak pušenja itd), ranijem početku i češćem skriningu, genetskom savjetovanju i genetskom testiranju.

Najdragocjenije i ujedno najjeftinije podatke za procjenu genetskog i ne genetskog raka pruža obiteljska anamneza koja treba obuhvatiti najmanje tri generacije svih članova obitelji (broj zdravih i bolesnih), etničko porijeklo ili rasu, dob pri dijagnozi i/ili smrti, uzrok smrti, vrstu raka i mjesto na kojem se nalazi razlikujući primarno mjesto i metastazu te navesti bilo koja prethodna oštećenja ili povezane bolesti (npr. nositelj ili bolesnik/bolesnica s ATM genom/uzročnik ataksije teleangiektazije).

Nadalje, bih se osvrnula na razlikovanje nasljedne sklonosti i stvarnog rizika pojave raka dojke i jajnika u slučaju prijenosa BRCA1 ili BRCA2 gena od jednog od roditelja, većinom od majke (koja češće ima rak dojke i/ili jajnika), vrlo rijetko od oca (rijetko boluje od raka dojke, prostate ili panktreasa). Ti se geni prenose na autosoman dominantan način, to jest 50% djece će imati gen, ali nužno neće biti bolesno, za razliku od drugih monogenskih bolesti.. Vjerojatnost da će neka specifična mutacija izazvati nastanak raka označuje termin penetrantnost. U slučaju vrlo visoko penetrantnog mutiranog gena svi nositelji (100%) t.j. 50% djece će razviti rak. Geni predstavljaju predispoziciju, dok mnogi drugi faktori poput hormona, načina života, prehrane itd predstavljaju provokaciju ili izazov . Malignost nastaje uzajamnim djelovanjem predispozicije i provokacije. Ipak, čini se da genetske mutacije imaju utjecaj izvan dosega prijemljivosti. Postoje dokazi da ekspresija gena može predvidjeti:

- Rizik bolesti
- Recidiv i rizik preživljenja
- Obrazac ponovnog pojavljivanja
- Odgovor na terapiju

Poseban izazov predstavlja testiranje gena koji predstavljaju sklonost raku. Takve analize su do nedavno služile kao mjera prevencije raka u testirane osobe i ili njezinih bliskih članova obitelji. Otkrivanje PARP inhibitora

u liječenju bolesnica s tumorom ovarija i inaktivacijom BRCA1 ili BRCA2 gena otvara nadu za učinkovitijom terapijom tih bolesnica i onih s drugim genima sličnog molekularnog djelovanja.

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http://cancer.gov/

National Cancer Institute. Sadrži informacije za bolesnike i zdravstvene radnike o liječenju različitih vrsta raka.

Dio koji se tiče genetike raka:

http://www.nci.nih.gov/cancerinfo/prevention-genetics-causes/genetics

http://genetests.org

GeneTests. Sadrži popis kliničkih i istraživačkih laboratorija koji pružaju tesriranje raznih vrsta nasljednih sindroma povezanih s rakom

ODGOVORI NA PITANJA I OBJAŠNJENJE

1 c. Rodoslovlje je sukladno AD nasljeđivanju raka dojke i jajnika s premenopauznom pojavom raka dojke u dvije bolesnice i rakom jajnika u jedne. Rak je uzrokovan mutacijom BRCA1 ili BRCA2 gena. Ostali geni su povezani s nasljednom sklonošću prema slijedećim malignim bolestima:

   a. RET, MEN1- multipla endokrina neoplazija tip 2 i - multipla endokrina neoplazija tip1

   b. TP53,PTEN –Li-Fraumni i Cowden sindrom

   c.

   d. MLH1, MSH2-nasljedni ne polipozni rak debelog crijeva

   e. BRC, ABL1-kronična mieloidna leukemija

2 a. Osoba III, 7 mora biti nositelj mutacije jer je poveznica između umrle bolesne sestre III, 3 i bolesne kćeri IV, 7 ; ne pokazuje pentrantnost za nasljedni rak dojke i jajnika zbog svog spola. Kćerka IV,7 je imala rizik koji ne bi imala u slučaju 2c. Nasljedni rak dojke i jajnika nije zahvaćen imprintingom (2d). Kako je otac (III, 11) naslijedio patološku mutaciju od asimptomatske majke (II,3),nepenetrantnost gena u iste nije odgovorna (2d).

3. b. Svako testiranje na povećanu sklonost treba započeti s bolesnikom što isključuje odgovore 3a , 3 c.i 3 e. Osobe III, 4 i IV,7 imaju rak ali je osoba IV, 7 bolji izbor budući da je u prvom koljenu u srodstvu s IV, 6 i ima rak s ranijim početkom. Osim toga, osobu III,4 dijele od IV, 7 tri osobe u kojih se radilo o nepenetrantnosti te bi osoba III, 7 mogla predstavljati fenokopiju.

GENETIKA RAKA

Nives PEĆINA-ŠLAUS

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Cancer is caused by mutations in our genome and can be considered a genetic disorder in which the normal control of cell growth is impaired. Genetic basis of cancer are overwhelming because they include changes of genes involved in a variety of vital cellular processes: cell growth, proliferation differentiation, apoptosis, DNA repair mechanisms, cell mobility, angiogenesis, immune system. Cancer genetics or better said genomics is now one of the fastest expanding medical specialties today and vast knowledge has been gathered in order to develop novel and efficient therapeutic methods. Molecular and genetic basis of tumor development although still not completely understood, are nowadays explained in many aspects. We understand now that cancer is not a single disease but rather a collection of diseases with specific genetic profiles. Furthermore, we know that malfunctioning of specific signal transduction pathways are responsible for tumor formation and development. Novel scientific papers, especially those that bring results on system biology of tumors and large-scale analyses, often refer to the collection of the observed genetic changes as cancer genome landscapes. Nevertheless, the classical categorization of genes to oncogenes and tumor suppressor genes still applies. Oncogene is a normal cellular gene that, when activated by mutation, increases the selective growth advantage of the cell in which it resides. Former studies that dealt with oncogenes provided knowledge on cell biology of somatic genetic mutations, the research of hereditary carcinoma discovered the existence of tumor suppressor genes. This is a gene that needs to be inactivated by mutation or lost to increase the selective growth advantage of the cell in which it resides. Novel categorization introduce driver genes and their mutations. Driver gene is a gene that contains driver gene mutations or is expressed aberrantly in a fashion that confers a selective growth advantage. Passenger gene is a gene that has no direct or indirect effect on the selective growth advantage of the cell in which it occurred but is vital for clinical outcome. There are also gatekeeper genes that, when mutated, initiate tumorigenesis in specific tissue. Finally, it is important to mention that Wnt signaling pathway that our group at the Croatian Institute for brain research is studying as part of Croatian Science Foundation project also plays important roles in a variety of human cancers.

GENETSKA OSNOVA RAKA DOJKE I JAJNIKA

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Rak dojke je složena bolest koja je rezultat progresivne akumulacije mutacija u mnogim genima, genetske i epigenetske poremećene regulacije kritičnih gena i proteinskih (signalnih) puteva. Uz individulanu varijabilnost i u dobi dijagnoze i fenotipske ekspresije bolesti, možemo reći da je rak dojke sa genetičkog stajališta vrlo heterogeno oboljenje (noviji podaci govore da je barem 200 gena uključeno u nastanak raka dojke).

Prema danas prihvaćenoj klasifikaciji (deklaracija St Gallen 2013), utemeljenoj na imunohistokemijskim postupcima tumori dojke dijele na 4 osnovna tipa (luminalne, luminalne HER2+/-, neluminalne i slične bazalnom).

Najvažnija postignuća posljednjih godina u razumijevanju razvoja tumora dojke su zahvaljujući novim tehnologijama i velikim prospektivnim studijama dovela do molekularnog profiliranja pojedinih imunokemijskih tipova kojima se klasificiraju podtipovi (potvrđuju ekspresijski profili i prognostičke vrijednosti specifičnih gena koji koreliraju sa prognozom i ishodom bolesti). Tumori dojke koji su estrogen receptor (ER) pozitivni su najbrojniji i najraznovrsniji, a tumori sa amplificiranim HER2 su terapijski efikasni, napose trostruko negativni nemaju ekspresiju u ER, progesteron (PR) i HER2, najčešće su bazalni, imaju kemoterapiju kao opciju i često su vezani uz mutacije u genu BRCA1.

Molekularni putevi kroz koje geni, odnosno proteini koje oni kodiraju, vezani su za proliferaciju tumorskih stanica i apoptozu te mehanizme popravaka oštećenja. U tumorima dojke treba spomenuti 3 osnovna puta:

Signalni put PI3K/Akt

Signalni put Fosfatidilinozitol 3-kinaze (PI3K) uključuje onkogen PI3K, tumor supresor PTEN te membranske tirozin kinaze EGFR, IGF1R koje aktiviraju PI3K i prenose signal prema AKT (serin/treonin kinazi) kroz kaskade fosforilacija koje se u tumorima neprestano aktiviraju. Mutacije u tom cijelom putu najčešće su nađene u luminalnom tipu raka dojke. Naprotiv, mutacije u tumor supresoru PTEN češće su u bazalnim tumorima.

Put tumor supresora p53

Mutacije u tumor supresoru p53 česta su pojava u estrogen ovisnim tumorima, npr u luminalnom A tipu s ER+ niska je frekvencija mutacija u p53, a viša u luminalnom B tipu, uz gubitak aktivnosti ATM i amplifikaciju MDM2.

Stanični ciklus

Kontrolne točke staničnog ciklusa (kao mjesta mutacija i promjena) značajnije su zastupljene u bazalnim tumorima, pračene su promjenama u regulaciji puta Rb proteina te mutacijama u genima BRCA1 i BRCA2.

Nadalje, osim genetskih, razvoju raka doprinosi i heterogenost epigenetskih promjena. Metilacija u DNA može imati prognostičke značajke kao i ekspresija malih nekodirajućih RNA i mikro RNA (miRNA).

Sposobnost integriranja podataka kroz sve danas poznate platforme daje ključne spoznaje i mnogo šire definira podtipove na temelju raznovrsnih ekspresijskih profila unutar osnovne 4 skupine tumora dojke, od kojih svaka pokazuje molekulsku heterogenost.

Breast cancer is a complex disease that is a result of the progressive accumulation of mutations in many genes, the genetic and epigenetic disregulation of critical genes, and protein (signaling) pathways. With the individual variability in age of diagnosis and phenotypic expression of the disease, we can say that breast cancer from a genetic point of view is very heterogeneous disease (recent data suggest that at least 200 genes are involved in breast cancer). According to the currently accepted classification (Declaration of St. Gallen, 2013), based on immunohistochemistry methods breast tumors are divided into four main types (luminal, luminal HER2 +/- non-luminal and basal). The most important achievements in recent years in understanding the development of breast cancer, thanks to new technologies and large prospective studies led to the molecular profiling of certain types of immunoassays which classify subtypes (confirm the expression profiles, and the prognostic value of specific genes that correlate with prognosis and outcome of the disease). Breast tumors that are estrogen receptor (ER) positive are the most numerous and most diverse, and tumors with amplified HER2 therapeutically effective,of triple negative - not expressing ER, progesterone (PR), and HER2, the most common are basal, have chemotherapy as an option and often associated with mutations in BRCA1.

The molecular pathways through which genes and the proteins they encode are linked to tumor cell proliferation and apoptosis, and the mechanisms of DNA damage repair. In breast tumors are three main pathways:

Signaling pathway PI3K / Akt

Phosphatidylinositol 3-kinase (PI3K) involves PI3K oncogene, tumor suppressor PTEN,and membrane tyrosine kinases EGFR, IGF-1R that activate PI3K and AKT ,they transmitt signal (serine / threonine kinase) through a cascade of phosphorylations and in tumors are permanently activated. Mutations are commonly found in luminal breast cancer. On the contrary, mutations in the tumor suppressor PTEN are frequently in basal tumors.

Pathway of tumor suppressor p53

Mutations in tumor suppressor p53 are common in estrogen-dependent tumors, e.g. luminal A type with low ER is low frequency of mutations in p53, but higher is in luminal B type, with the loss of activity of ATM and amplification of MDM2.

Cell cycle control.

Control points of cell cycle (as a points of mutations and changes) significantly are represented in the basal tumors, followed with changes in regulation of the Rb protein, and mutations in the genes BRCA1 and BRCA2.

Furthermore, in addition to genetic, epigenetic changes also contribute to cancer development and particularly heterogeneity of epigenetic changes. Methylation of the DNA can have prognostic features as the expression of small non-coding RNA and micro RNA (miRNA).

The ability to integrate data through all today known platforms provides key insights and more broadly defined subtypes on the basis of a variety of expression profiles within the main mentioned 4 types of breast tumors, each of which shows the molecular heterogeneity.

DESET GODINA TESTIRANJA BRCA1 I BRCA2 MUTACIJA U HRVATSKOJ

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SAŽETAK

Rak dojke je najčešće maligno oboljenje kod žena i drugi najčešći uzrok smrti povezane s rakom. Najmanje 10 % slučajeva pripisuje se obiteljskim utjecajima. U Hrvatskoj, svake godine dijagnosticira se više od 2500 slučajeva raka dojke, dok preko 900 žena od njega i umire.

Rak jajnika je najsmrtonosnije ginekološko oboljenje, uglavnom zbog uznapredovalog stupnja i visokog gradusa kod otkrivanja bolesti. U Hrvatskoj je rak jajnika otprilike na šestom mjestu po učestalosti, te od njega godišnje oboljeva oko 450 žena, dok ih oko 330 umire.

BRCA1 i BRCA2 su glavni geni odgovorni za nasljedni rak dojke i jajnika. Žene nositeljice mutacija u genima BRCA1 ili BRCA2 imaju rizik od 45-85 % nastanka raka dojke, te rizik od 11-39 % nastanka raka jajnika do dobi od 70 godina. Mutacije u bilo koja od ta dva gena su povezane s nastankom i sporadičnog i nasljednog raka dojke i jajnika. U slučaju nasljednog raka, osoba nasljeđuje jednu mutiranu kopiju bilo kojeg od ta dva gena. Mutirana kopija se može naslijediti s bilo koje strane obitelji. Tumorigeneza nastaje kad uz već postojeću mutiranu kopiju, osoba razvije inaktivirajuću mutaciju preostalog, zdravog alela.

U Hrvatskoj je istraživanje mutacija u genima BRCA počelo prije otprilike deset godina. Dosad je analizirano gotovo 500 osoba, oko 220 zdravih kontrola i oko 240 osoba s nasljednom sklonosti nastanku raka dojke i jajnika.

Cjelokupna kodirajuća regija i rubovi introna oba gena analizirani su metodom krivulje mekšanja visoke rezolucije, direktnim sekvenciranjem i metodom semi-kvantitativnog multipleks PCR-a. Na taj način se mogu detektirati i male mutacije i veliki rearanžmani.

Dosad je otkriveno 17 patogenih mutacija, 10 u genu BRCA1 i 7 u genu BRCA2. Jedna od mutacija u genu BRCA1 (c.5335C>T) i tri mutacije u genu BRCA2 (c.4139_4140dupTT, c.8175G>A i c.3925delA) nisu dosad bile opisane, ali su dvije od njih nedavno otkrivene u Srbiji i Sloveniji. Osim patogenih mutacija, nađeno je i 27 BRCA1 i 55 BRCA2 polimorfizama i promjena nepoznatog značaja, od kojih 4 u BRCA1 i 14 u BRCA2 dosad nisu bile objavljene.

KLINIČKA DIJAGNOSTIKA I INDIKACIJE ZA GENETSKO TESTIRANJE RAKA DOJKE I JAJNIKA

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SAŽETAK

Rak dojke najčešće je sijelo raka u žena u praktički svim područjima svijeta. U Europi od raka dojke obolijeva godišnje gotovo 460.000 žena (29% novooboljelih žena). Rak jajnika je 5. najčešće sijelo raka u žena u Europi, s preko 65.000 novih slučajeva godišnje. Na nasljedni rak dojke otpada oko 10-15 % svih slučajeva raka dojke. Već dvadesetak godina su poznate mutacije u dva gena tumorska supresora, gena BRCA1 i BRCA 2 (od engl breast cancer gene 1, breast cancer gene 2. Osobe s naslijeđenim mutacijama u jednom od ta dva gena tijekom života imaju veću vjerojatnost razviti rak dojke i/ili jajnika od opće populacije. Dvije su skupine unutar kojih ćemo izdvojiti pojedince koje ćemo uputiti u genetsko savjetovalište, a potom eventualno i genetski testirati. Prva skupina su osobe oboljele od raka dojke ili jajnika koje zadovoljavaju BAREM JEDAN od sljedećih kriterija: oboljela od raka dojke prije 50. godine života, „trostruko negativni" rak dojke (ER -, PR-, HER2-) prije 60. godine života, bilateralni ili multicentrični rak dojke, ima barem jednu blisku srodnicu koja je oboljela od raka dojke prije 50. godine života, ima barem jednu blisku srodnicu koja je oboljela od raka jajnika, dva ili više bliska srodnika oboljela od raka dojke i/ili gušterače u bilo kojoj dobi, ako u osobnoj ili obiteljskoj anamnezi ima barem tri od sljedeće navedenog: rak gušterače, rak prostate (Gleason score jednak ili veći od 7), sarkom, rak nadbubrežne žlijezde, tumore mozga, rak maternice, rak štitnjače, rak bubrega, kožne promjene i/ili makrocefaliju, polipe u probavnom sustavu, difuzni rak želuca; zatim pripadnost etničkim skupinama s visokom učestalošću mutacija (npr Aškenazi Židovi) i bez pozitivne obiteljske anamneze, zatim ako se radi o oboljelom od raka dojke muškog spola, te ako u obitelji postoji osoba koja je nositelj mutacije gena za nasljedni rak dojke i jajnika, te žene koje imaju ili su imale invazivni rak jajnika / jajovoda / peritonealnog karcinoma. Zdrave osobe koje zadovoljavaju BAREM JEDAN od sljedećih kriterija: osoba u obitelji ima srodnika koja je nositelj mutacije gena za nasljedni rak dojke i jajnika, ako ima barem dvije bliske srodnice s rakom dojke, ako ima barem jednu blisku srodnicu s rakom jajnika, ako ima barem jednu blisku srodnicu s rakom dojke koja je oboljela prije 45 godine života, ako u osobnoj ili obiteljskoj anamnezi ima barem tri od sljedeće navedenog: rak gušterače, rak prostate (Gleason score jednak ili veći od 7), sarkom, rak nadbubrežne žlijezde, tumore mozga, rak maternice, rak štitnjače, rak bubrega, kožne promjene i/ili makrocefaliju, polipe u probavnom sustavu, difuzni rak želuca, ako u obitelji ima osobu koja je oboljela od raka dojke, a muškog je spola. Ispunjavanjem jednog ili više od gore navedenih kriterija potrebno je učiniti personaliziranu procjenu rizika, genetsko savjetovanje, te potom eventualno testirati. Testiranje zdravog pojedinca se radi samo onda kada nam za testiranje nije dohvatljiv prikladan oboljeli bliski srodnik.

CLINICAL DIAGNOSTIC AND GENETIC TESTING FORBREST AND OVARIAN CANCER

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ABSTRACT

Breast cancer is the most common cancer in women worldwide. In Europe there are almost 460 000 of new cases annually (29% of all new cancer cases). Ovarian cancer is 5th most common cancer site in women in Europe with more than 65 000 new cases annually. Hereditary breast cancer comprises 10-15% of all breast cancers. Mutations in two tumour suppressor genes are well known for almost two decades, BRCA 1 and BRCA 2 (from breast cancer gene 1 and breast cancer gene 2). Person with inherited mutation in one of these genes has greater probability for developing breast and/or ovarian cancer than person in general population. There are two groups from which we pick individuals for genetic counselling and testing. The first group are affected individuals with breast or/and ovarian cancer with at least one of the following criteria: breast cancer before the age of 50; triple negative breast cancer before the age of 60; bilateral or multicentric breast cancer; at least one relative with breast cancer before the age of 50; at least one relative with ovarian cancer; two or more close relatives with breast and/or pancreatic cancer in any age; personal or family history of at least three of the following criteria: pancreatic cancer, aggressive prostate cancer, sarcoma, suprarenal gland cancer, brain cancer, uterine cancer, thyroid cancer, renal cancer, skin changes and/or macrocephaly, gastrointestinal tract polyps, diffuse gastric cancer; origin from ethnic groups with high rates of BRCA mutations; male with breast cancer; breast and/or ovarian cancer mutation carrier in family; women with invasive ovarian/Fallopian tube/peritoneal carcinoma. The second group are healthy individuals with at least one of the following criteria: breast and/or ovarian cancer mutation carrier in family; at least two close relatives with breast cancer or one with ovarian cancer; at least one relative with breast cancer before the age of 45; personal or family history of at least three of the following criteria: pancreatic cancer, aggressive prostate cancer, sarcoma, suprarenal gland cancer, brain cancer, uterine cancer, thyroid cancer, renal cancer, skin changes and/or macrocephaly, gastrointestinal tract polyps, diffuse gastric cancer; one close relative with male breast cancer. Fulfilling one or more criteria listed above warrants further personalized risk assessment, genetic counselling, and, often, genetic testing and management. Testing of healthy individuals should only be considered when an appropriate affected family member is unavailable for testing.

GENETSKO SAVJETOVANJE PRIJE I POSLIJE TESTIRANJA NA NASLJEDNI RAK DOJKE I JAJNIKA

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SAŽETAK

Genetsko savjetovanje je komunikacijski proces koji se bavi genetskim poremećajima ili rizikom pojave genetskih poremećaja unutar obitelji. To je multidisciplinarni specijalizirani postupak koji sadrži dvije osnovne komponente: racionalnu (kognitivnu) i emocionalnu. Proces genetskog savjetovanja podrazumijeva nastojanje adekvatno educirane osobe da pomogne jednoj ili više osoba unutar obitelji da: razumije medicinske činjenice o genetskom poremećaju, razumije kako genetski čimbenici utječu na pojavnost bolesti unutar obitelji, razumije mogućnosti djelovanja s obzirom na specifični rizik pojave genetskog poremećaja, odabere i provede za sebe najprikladniji način djelovanja s obzirom na osobni rizik pojave genetskog poremećaja, razumije i prihvati ponuđenju informaciju s ciljem promocije zdravlja, smanjenja psihološkog distresa i unapređenja brige o sebi, se na najbolji mogući način prilagodi pojavi genetskog poremećaja s obzirom na osobni rizik.

Genetski savjetnik vodi računa o psihološkom stanju i emocionalnoj reakciji osobe koja treba razumjeti saopćenje kako bi mogla donijeti odluku koja je najbolje za nju.

Genetsko savjetovanje mora biti dobrovoljno, odnosno osoba ima pravo ostati neinformirana. Testiranje bez genetskog savjetovanja može imati dalekosežne posljedice, s obzirom da je njegova osnovna zadaća da donese dobrobit i umanji, ili posve ukloni, moguće štetne posljedice rezultata genetskog testiranja.

Prema Europskoj konvenciji o ljudskim pravima i biomedicini, donesenoj 4. travnja 1997. godine u Oviedu, genetskom testiranju mora prethoditi primjereno genetsko savjetovanje.

Hrvatska je 2003. godine ratificirala Konvenciju, a radi usklađivanja hrvatskog zakonodavstva s odredbama Konvencije, u studenome 2004. godine izglasan je Zakon o zaštiti prava pacijenata.

Prvim razgovorom i pregledom u genetskom savjetovalištu, prikupljaju se informacije važne za medicinski problem zbog kojeg je osoba došla na konzultaciju. Uzima se detaljna obiteljska i osobna anamneza (uključujući psihičke poremećaje), sastavlja obiteljsko stablo, obavlja ciljani klinčki pregled, te se osobu informira o prirodi bolesti, mogućnostima prevencije, rane dijagnoze i liječenja, načinu nasljeđivanja, riziku pojave bolesti u osobe koju se savjetuje, pouzdanosti i ograničenjima genetskog testa koji se razmatra, mogućim psihološkim implikacijama, te drugim mogućim posljedicama na osobu koju se savjetuje, odnosno na članove obitelji te osobe. Naglašava se privatnost i povjerljivost podataka, a savjetovanje se obavlja prema načelu nedirektivnosti. Osobi koju se savjetuje, treba ponuditi dovoljno vremena da za sebe, na temelju dobivenih informacija, donese najbolju moguću odluku. Ukoliko osoba želi, genetski savjetnik joj uručuje kratak pisani dokument nakon prvog razgovora.

Genetsko savjetovanje poslije genetskog testiranja potrebno je i u slučaju pozitivnog, negativnog ili neinformativnog rezultata testa. Prigodom priopćavanja rezultata potrebno je djelomice ponoviti objašnjenja koja su prethodila uzimanju uzorka. Informaciju o rezultatima testiranja treba pružiti na jasan način, vodeći računa o mogućim emocionalnim reakcijama. Nakon priopćavanja rezultata genetskog testiranja, najvažnije je procijeniti na koji je način osoba interpretirala rezultat testiranja, jer daljnje psihološke reakcije i ponašanja ovise primarno o percipiranoj, a ne dobivenoj informaciji. Stoga genetski savjetnik treba provjeriti kako je osoba shvatila infromaciju i u slučaju krive interpretacije treba suosjećajno, ali jasno upozoriti osobu na krive zaključke te joj ponuditi dodatne infromacije.

Procjenjuje se potreba eventualnog genetskog savjetovanja i/ili genetskog testiranja članova obitelji.

Stvara se medicinski dom za ispitanika i članove obitelji pod rizikom, te se kroz genetsko savjetovalište omogućuje kontakt sa drugim specijalistima uključenim u proces praćenja i liječenja.

Ovisno o procjeni razumijevanja dobivenih infromacija, te psihološke reakcije procjenjuje se potreba za nastavkom genetskih konzultacija, odnosno za psihološkim savjetovnjem.

GENETIC COUNSELING BEFORE AND AFTER GENETIC TESTING FOR HEREDITARY BREAST AND OVARIAN CANCER

SUMMARY

Genetic counseling is a communication process that deals with genetic disorders or risks of genetic disorders in the family. This is a multidisciplinary specialized procedure that consists of two basic components: rational (cognitive) and emotional. The process of genetic counseling involves the effort of adequately trained person to help the others in the family to: understand the medical facts about the genetic disorder, understand how genetic factors affect the incidence of the disease in the family, understand the possibilities of action with regard to the specific risk of a genetic disorder, and to choose the most appropriate course of action with regard to the personal risk of genetic disorder, to understand and accept the information to promote health, reduce psychological distress and improve self-care, in the best possible way due to personal risk.

Genetic counselor takes into account the psychological status and emotional reaction of the person who needs to understand the statement in order to make a decision that is best for her.

Genetic counseling should be voluntary, and person has the right to remain uninformed. Testing without genetic counseling could have far-reaching implications, because its main task is to bring well-being and reduce, or completely eliminate, the potential adverse consequences of the results of genetic testing.

According to the European Convention on Human Rights and Biomedicine, accepted on 4th of April in 1997 in Oviedo, genetic testing must be preceded by appropriate genetic counseling.

In 2003, Croatia ratified the Convention, and harmonized Croatian legislation with the provisions of the Convention in November 2004 by passing the Law on the Protection of Patients' Rights.

In the first interview, the information important medical problem for which the person came to the consultation is collected. A detailed family and personal history (including mental disorders) is taken, a family tree is constructed, the person is examined and informed about the nature of the disease, possible prevention, early diagnosis and treatment, mode of inheritance, the risk of occurance of the disease in the person that is advised, reliability and limitations of genetic test are taken under consideration so as possible psychological implications and other possible consequences. Privacy and confidentiality are emphasized, and counseling is done according to the principle of indirect access to the patient. The person that is advised, should have enough time to bring the best possible decision, based on the information obtained. If a person agrees, genetic counselor can write a brief document after the first interview.

Genetic counseling after genetic testing is necessary in the case of a positive, negative or uninformative test results. Sometimes, the explanations preceding the sampling should be partly repeated. Information must be provided in a clear manner, taking into account the possible emotional reactions. After reporting the results of genetic testing, the most important is to assess the way to which people interpret test result, as further psychological and behavioral reactions depend primarily on the perceived rather than the information obtained. Therefore, the genetic counselor should check that the person understood the information properly and in the case of misinterpretation should be sympathetic, and clearly warn the person to the wrong conclusions and offer her the additional information.

The need for possible genetic counseling and / or genetic testing of family members is estimated.

A medical home for patients and family members at risk is to be created, and contact with other specialists involved in the process of monitoring and treatment is enabled.

Depending on individual psychological reaction, the need for continuing genetic consultation, or for psychological counceling is estimated.

THE ROLE OF THE PATHOLOGIST IN THE DIAGNOSIS AND TREATMENT OF BREST AND OVARIAN CANCER

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Breast cancer is the most common cancer among women in both developing and developed regions in the world. Clinical cancer develops over a long period of time, requires multiple molecular alterations, and involves evolution of cellular populations with increasingly aggressive phenotypic characteristics. Although the time required for the process of carcinogenesis is not well established for any human cancer, estimate suggest that this multistep process unfolds over many years and possibly several decades. Breast cancer represents a diverse collection of malignant diseases of the breast with highly variable clinical behaviors and disparate response to therapy.

Personalized oncology is evidence-based, individualized medicine that delivers the right care to the right cancer patient at the right time and results in measurable improvements in outcomes and a reduction on health care costs. The essence of personalized oncology lies in the use of biomarkers. Also, they have different importances: predictive, prognostic and early response biomarkers.

The diagnosis and treatment of breast cancer has rapidly evolved over the past 20 years. In the first part of the 20th century, treatment of breast cancer consisted of radical mastectomy, but adjuvant systemic treatment and adjuvant radiotherapy did not play a major role. Diagnosis of breast cancer was mostly made based on clinical presentation, later aided by mammography and often combined with frozen section pathology confirmation. Starting in the 1980s, there have been important alterations in the diagnosis and treatment of breast cancer, having an important impact on the diagnostic procedure employed by pathologists.

Histopathological features play an important role in guiding the treatment decisions. In addition, genetic research is starting to have an increasing impact on guiding therapy by providing prognostic and predictive factors.

To obtain optimal morphology in the histology sections, and to obtain optimal immunohistochemical staining results, the resection specimen should be cut into thin slices immediately after surgery.

For microscopic examination the pathologist should be obtained and processed for paraffin sections full diameter of the tumour and its surroundings, small part of the tumour to perform immunohistochemistry, if there are macroscopical or radiological abnormalities in the tissue surrounding the invasive tumour, these areas should be sampled. If the surrounding tissue is without abnormalities, it is necessary to take at least two sections from macroscopically normal breast tissue.

On slides stained with hematoxylin eosin (H.E.), pathologist must determine the prognostic and predictive factors for breast cancer. This includes the histological type of cancer, the degree of tumour differentiation, the degree of nuclear differentiation, mitotic counts, lymphovascular invasion, estrogen and progesterone receptors, protein HER-2, proliferative index Ki-67, status of the axillary lymph nodes and the largest tumor deposit in the lymph node.

Receptors are determined by immunohistochemistry and the results are expressed as the percentage of positive cells and intensity of staining. Staining for estrogen and progesterone receptor is allways nuclear in localization and in most institutes all patients with a tumour in which more than 10% or more 1% of the tumour cells show positive staining regardless of the intensity of staining are candidates for adjuvant hormonal therapy. According to the consensus of the St Gallen 2014. cut-off of the progesterone receptors is 20%. This value best separating luminal type A from luminal type B breast cancer. Values below 20% indicate that the progesterone receptors are negative or low. When negative staining for estrogen and/or progestrone receptor is seen, it is important to confirm that staining of the hormone receptor-negative case has been successful. This can usually be tested, since the majority of normal breast tissues contain some nuclei ducts and lobules that are positive for estrogen

and progesterone receptor. If no normal breast epithelial cells are found to show positive staining, the hormone receptor assays should be repeated on another tumour block.

HER-2 gene amplification is observed in 15-30% of invasive breast cancers and leads to HER-2 receptor over-expression. HER-2 positive invasive breast cancers respond favourable to therapies that specifically target the HER-2 protein, therefore it is very important today to identify candidates for this type of targeted therapy. Several technologies are available for determining HER-2 status, but the two most commonly used are immunohistochemistry (IHC), which measures HER-2 protein expression and SISH (silver in situ hybridisation) which detects HER-2 gene amplification a method that is often used today in the pathology than FISH (fluorescence in situ hybridisation). The interpretation of the results is based on the intensity and percentage of stained cells. The most commonly used score system is 0, 1+ (negative results) , 2+ and 3+ (positive results). A 2+ is considered equivocal and should be followed by retesting by SISH. Women with IHC 3+ tumours are candidates for therapy with trastuzumab, but women with 2+ tumour should be retested and if the results show amplification of gene of those are candidates for trastuzumab. To ensure the highest possible accuracy, pathology centers must standardise methodologies and testing procedures.

Proliferative index is also very important and is determined by immunohistochemistry by monoclonal antibody Ki-67. Positive reaction is nuclear reaction and are counted positive nuclei in 1000 tumour cells on the high magnification and the results obtained is expressed as a percentage of positive nuclei. According to St Gallen consensus cut of value is 20% of positive cells, which means that below this value is low and value above 20% is high proliferative index.

Based on the receptors, HER-2 status and proliferative index breast cancers are classified immunophenotypically into five subgroups: luminal type A, luminal type B HER-2 negative, luminal type B Her-2 positive, HER-2 positive (non-luminal type) and triple negative tumours. Based on the immunophenotype of the cancer patients receive appropriate therapy. The multi-gene testing remains inaccesible for the majority of women with early breast cancer, therefore is adopted clinico-pathological testing, now expressed in surrogate IHC-based classification.

In order to apply conserving surgery or perhaps in some cases even avoided, introduced a minimally invasive biopsy changes in the breast biopsy (core needle biopsy). The samples were cylinders of tissue in which the pathologist must diagnose and determine many of the above prognostic and predictive factors for the application of neoadjuvant and adjuvant therapy. Changes in the tissue after treatment are numerous and that the pathologist must know. He must determine the effect of therapy and determine whether there is a presence of a tumor after treatment or there is a complete pathological response in both the primary tumor and the affected lymph node metastases.

Numerous studies in recent years have identified many prognostic and predictive factors for breast cancer. Most of them determined pathohistologically, which resulted in a large responsibility for pathologists. In addition, the pathologist has become a key person in a multidisciplinary team of breast cancer and the person very responsible for the implementation of specific individual therapy.

Ovarian cancer patients interact with many doctors during the course of their treatment, but rarely do they meet the specialist who plays a critical role in the outcome: the pathologist who diagnoses their cancer by analyzing samples of tissue. Precise diagnosis is what drives patient decisions and therapy. If pathology is wrong, everything that follows will likely be incorrect as well. Cases of suspected and confirmed advanced stage ovarian cancer should be discussed by multidisciplinary team and pathologist is one the key person within the team. Never before in history have pathologist been so critically important.

Core histological data are tumor type, tumor grade, microinvasion, lymph nodes status, peritoneal biopsies, omentum, fallopian tubes and staging.

The tumour should be designated according to the World Health Organization (WHO) classification. Epithelial ovarian tumours are heterogeneous neoplasms which are primarily classified according to cell type into serous, mucinous, endometrioid, clear-cell, transitional and squamous cell tumours. More importantly, these tumours

are further subdivided into benign, borderline (intermediate), and malignant (carcinoma) depending on the degree of cell proliferation and nuclear atypia, and the presence or absence of stromal invasion.

Currently, based on histopathology, immunohistochemistry, and molecular genetic analysis, at least five main types of ovarian carcinomas are identified: high-grade serous carcinomas (HGSC), endometrioid carcinoma (EC), clear cell carcinomas (CCC), mucinous carcinomas (MC) and low-grade serous carcinomas (LGSC).

According to recent study serous carcinoma is low (LGSC) or high grade (HGSC). They are fundamentally different tumour types, and consequently different diseases. LGSCs are associated in most cases with a serous borderline component, carry KRAS and BRAF mutations, and are unrelated to p53 mutations and BRCA abnormalities. In contrast, HGSCs are not associated with serous borderline tumours and typically exhibit p53 mutations and BRCA abnormalities

Mucinous carcinoma are graded in a similar manner to endometrioid carcinoma, as is done in the uterus. Recently, mucinous carcinomas have been divided into two categories:an expansile type without obvious stromal invasion, but exibiting back-to back or malignant glands with minimal or no intervening stroma, and exceeding 10mm2 in area and an infiltrative type showing evident stromal invasion. The expansile pattern of growth is associated with a more favorable prognosis than the infiltrative pattern.

Endometrioid carcinomas are graded as I,II or III using the FIGO grading system which is used for the grading of uterine endometrioid adenocarcinomas.

Ovarian clear cell carcinomas and transitional cell carcinomas are regarded as automatically high grade or grade III.

Microinvasion may occur within an otherwise typical borderline tumour, usually of serous or mucinous type. Microinvasion has been found to have no adverse effect on prognosis and may be multifocal. If the foci of microinvasion are clearly separate, these can be regarded as multiple distinct foci of microinvasion and the size of separate foci need not be added together.

The total number of lymph nodes examined from each anatomical site and the number involved by tumour shoul be recorded.

The presence or absence of tumour involvement in biopsies from each anatomical site should be recorded. Peritoneal involvement in association with an ovarian borderline tumour, especially of serous type, may take form of invasive or non-invasive implants which may coexist. The lesions were confined to the surface of organs are non-invasive implants or infiltrated the underlying tissue are invasive. This is a difficult area and may require specialist internal or external review.

The size of the largest omental metastatic deposit should be documented. Omental involvement in association with a borderline tumour, especially of serous type, may take the form of invasive or non-invasive implants. Since invasive and non-invasive implants may, on occasions, coexist and since invasive implants are associated with an adverse prognosis are an indicator for adjuvant chemotherapy, extensive omental sampling should be undertaken when non-invasive implants are identified in the original sections

The presence or abscence of tubal involvement should be documented as well as site of tubal involvement, for example mucocal or serosal. Tubal involvement in ovarian carcinoma is not uncommon and the fimbria is the most common site. It has, in fact been suggested that the tubal fimbria is the site of origin of many pelvic serous carcinoma. It is now accepted that a number of what have been thought to be primary ovarian cancer are originated in other pelvic organs and involve the ovary secondarily.

Tumours should be staged according to the FIGO staging systems. Although it is useful to record the provisional stage on the histopathology report, the final stage should be determined at the multidisciplinary team meeting where the results of all clinical, radiological and pathological parameters can be correlated.

Never before in history have pathologist been so critically important. Therefore, pathologist needs some clinical information, and the specimen request form should include full patient details and the results of any previous biopsy or cytology specimens.

Sometimes radiologically guided core biopsies are performed to confirm the diagnosis preoperatively or prior to chemotherapy or in patients who are too ill to undergo a laparotomy. The number of core biopsies should be stated and the length of each core documented. Tissue may need to be preserved so that a range of immunohistochemical markers can be performed. Materials received with core biopsies are small biopsy specimens and pathologists need to be highly qualified and experienced in gynecological pathology.

Intraoperative pathological consultation with frozen section is of value in cases where clinical management decisions may be altered depending on the histological type and grade of tumour, e.g. young women for whom continuing fertility is crucial. Situations where frozen section examination might be performed include:

- intraoperative assessment of a neoplasm confined to the ovary to assess whether this is benign, borderline or malignant; this may direct whether lymphadenectomy or other staging procedures are undertaken
- for confirmation of an epithelial neoplasm, for subtyping of an epithelial malignancy and, in cases of obvious malignancy to distinguish between a primary ovarian and a metastatic neoplasm

Clinicians should be aware that a single sample may be not provide adequate material for the histopathologist whereas, further sampling for paraffin sections may result in upgrading of a frozen section diagnosis of benign to borderline tumour or of high grade borderline tumour to invasive carcinoma. If any doubt is expressed by the pathologist in frozen section, the more conservative diagnosis must be the „working" diagnosis for immediate patient management.

Immunohistochemistry has many applications in the field of ovarian neoplasia and the use of immunohistochemistry has significantly increased in recent years. The results of any immunohistochemical stains should always be carefully interpreted in conjunction with the clinical, gross and microscopic features. However, areas where immunohistochemistry may contribute significantly include the following:

- distinction between a primary ovarian adenocarcinoma and metastatic adenocarcinoma from various sites (potentially useful markers include cytokeratin 7 and 20, CA 125, CEA, CA 19.9, WT 1, TTF-1, oestrogen receptor and CDX 2)
- typing of an ovarian adenocarcinoma, most ovarian serous carcinoma exhibit nuclear positivity with WT 1, while most of the other morphological subtypes are negative
- the distinction between an epithelial and a sex cord-stromal tumour (potential useful markers include inhibin, calretinin positive in sex cord-stromal tumours and epithelial membrane antigen- EMA and cytokeratin 7 positive in epithelial neoplasms)

Many advances of molecular genetics of ovarian cancer have been made, but these are not yet affecting clinical practice except BRCA 1 and BRCA 2 genes. Ovarian cancer mostly arises sporadically, but a fraction of cases are associated with mutation in BRCA 1 and BRCA 2 genes. The presence of BRCA mutations in ovarian cancer patients, especially in patients with HGSC has been suggested as a prognostic and predictive factor. Tumour pathological data are very important for the molecular analysis and should be included in the results of molecular testing.

ULOGA PATOLOGA U DIJAGNOSTICI I TERAPIJI RAKA DOJKE I JAJNIKA

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Unazad 20 godina dijagnostika i liječenje karcinoma dojke snažno je napredovalo. Počevši od 1980. godine kada su počele biti važne promjene u dijagnostici i liječenju karcinoma dojke, sve je to utjecalo na dijagnostički postupak i rad patologa. Brojna novija istraživanja utvrdila su većinu prognostičkih i prediktivnih čimbenika za karcinom dojke. Većina njih određuje se patohistološki bilo standardnom histološkom metodom a što znači uzorci tkiva obojeni hemalun eozinom bilo imunohistokemijski. Sve to je u konačnici rezultiralo velikom odgovornošću patologa koji je postao ključna osoba u multidisciplinarnom timu za karcinom dojke i vrlo odgovorna osoba za primjenu specifične individualizirane terapije.

Tradicionalni prognostički i prediktivni čimbenici su životna dob, veličina tumora, status limfnih čvorova pazuha, histološki tip, stupanj diferenciranosti tumora i jezgre, peritumorska limfovaskularna invazija i status hormonskih receptora. Spomenuti čimbenici imaju manjak podataka o biološkoj različitosti karcinoma dojke i ne odražavaju kompleksnost molekulskog mehanizma bolesti, no još uvijek su dobri za kliničku odluku o odgovarajućoj terapiji. Veličina tumora, histološki tip, stupanj diferenciranosti tumora i jezgre kao i stadij tumora su vrlo snažni tradicionalni prognostički i prediktivni čimbenici za oboljele od karcinoma dojke, premda su metode za njihovo mjerenje teško standardizirane. Peritumorska limfovaskularna invazija važan je pokazatelj rizika za lokalnu i udaljenu pojavu bolesti i neposredno je povezana sa zahvaćanjem lokoregionalnih limfnih čvorova. Dokazana je njezina povezanost i prognostička vrijednost u praćenju tijekom dugog vremenskog razdoblja. Važnost određivanja limfovaskularne invazije je njezina karakteristika kao snažnog prediktivnog čimbenika u slučaju poštedne terapije.

Spoznaja da je oko 30% karcinoma dojke hormonski ovisno i da ima pozitivne steroidne receptore, rezultirala je njihovim određivanjem u prvo vrijeme u citosolu tumorskog tkiva a vrijednosti su bile izražene u femtomolovima. Razvoj monoklonskih protutijela specifičnih za proteine za estrogenske (ER) i progesteronske (PR) receptore omogućio je uvođenje imunohistokemijske metode u određivanju ER i PR u karcinomu dojke. Danas patolog u svom nalazu mora iskazati postotak pozitivnih tumorskikh stanica i intenzitet obojenja. Prema prihvaćenim smjernicama St.Gallena iz 2014. godine prijelomna vrijednost za progesteronske receptore je 20%. Spomenuta vrijednost najbolje odjeljuje luminalni tip A od luminalnog tipa B karcinoma dojke. Vrijednosti progesteronskih receptora do 20% znače da su oni negativni ili niski. Kao kontrola uspješnosti reakcije na steroidne receptore koristi se okolno zdravo tkivo u kome se nalaze normalni izvodni kanali parenhima dojke i koji daju pozitivnu reakciju na steroidne receptore. Nalaz je vrlo važan kako za određivanje imunofenotipa karcinoma dojke tako i za primjenu hormonske terapije u liječenju karcinoma dojke. S obzirom da je estrogenski receptor termolabilan i da ima vrlo brzo vrijeme poluraspada, kirurški odstranjeni uzorak tkiva dojke s tumorom potrebno je u što kraćem vremenskom periodu dostaviti na patologiju kako bi se što prije uzeo u obradu te kako bi se na taj način smanjila mogućnost gubitka receptora a što u konačnici može rezultirati i nižim vrijednostima receptora a da to nije zapravo prava vrijednost.

Amplifikacija gena HER-2 uočena je u oko 15-30% invazivnih karcinoma dojke što rezultira pojačanom izraženošću receptora za HER-2. Pojačana izraženost HER-2 u bolesnica s invazivnim karcinomom dojke omogućava primjenu specifične terapije te je danas vrlo važno odrediti koje su bolesnice kandidati za specifičnu terapiju odnosno primjenu Herceptina ili trastuzumaba. Postoji nekoliko metoda za određivanje HER-2 statusa, no najčešće se primjenjuju imunohistokemijska metoda kojom se određuje izraženost proteina HER-2 i SISH (silver in situ hibridizacija) kojom se određuje amplifikacija gena. Rezultati analize određuju se na temelju intenziteta obojenja i postotka pozitivnih stanica. Reakcija 0 i 1+ su negativni rezultati, 2+ nalaz mora biti retestiran metodom SISH kako bi se vidjela razina amplifikacije gena. Ukoliko je taj nalaz pozitivan tek je tada bolesnica kandidat za specifičnu terapiju. Spomenuta metoda može se raditi samo u visoko specijaliziranim centrima čiji su laboratoriji proveli kontrolu metode. Reakcija s oznakom 3+ je pozitivna i bolesnica dobiva specifičnu terapiju.

Proliferacijski biljezi upotrebljavaju se kako bi se odredio broj stanica u diobi te kako bi se na taj način vidjelo kakvo je biološko ponašanje tumora odnosno bolje rečeno kakav će biti odgovor na primijenjenu terapiju. Danas se rutinski u patohistološkoj dijagnostici proliferacijski indeks određuje upotrebom monoklonskog protutijela Ki-67. Metodom se određuje postotak pozitivnih stanica odnosno jezgara na 1000 tumorskih stanica na 10 vidnih polja najvećeg povećanja mikroskopa. Prijelomna vrijednost koja označava nizak odnosno visok proliferacijski indeks je Ki-67 20%. Ova vrijednost važna je za određivanje imunofenotipa tumora a posljedično i najvažnija za određivanje terapije.

Temeljem nalaza steroidnih receptora, HER-2 statusa i proliferacijskog indeksa karcinom dojke se imunofenotipski klasificira u pet podtipova: luminalni tip A, luminalni tip B HER-2 negativan i luminalni tip B HER-2 pozitivan, HER-2 tip (neluminalni tip) i trostruko negativan. Prema imunofenotipu tumora bolesnice dobivaju adekvatnu terapiju. S obzirom da su multigenske metode testiranja skupe i većini žena s ranim karcinomom dojke danas još uvijek nedostupne u upotrebi su kliničko-patološka testiranja izražena kao IHC (imunohistokemijska) zamjenska klasifikacija imunofenotipa karcinoma dojke.

Tijekom vremena mijenjao se i kirurški pristup liječenja bolesnica oboljelih od karcinoma dojke što je u novije vrijeme rezultiralo sve većom primjenom poštednih kirurških zahvata. Kako bi se mogao primijeniti što poštedniji kirurški zahvat ili možda u nekim slučajevima čak i izbjeći, uvedena je i minimalno invazivna biopsija promjena u dojci odnosno biopsija širokom iglom (engl. core needle biopsy). Na taj način dobiveni uzorci tkiva izgleda su poput cilindra i promjera ovisno o promjeru igle s kojom se uzima tkivo . Patolog na tako dobivenim uzorcima tkiva mora postaviti dijagnozu i odrediti sve prethodno navedene prognostičke i prediktivne čimbenike ovisno o tome da li je bolesnica kandidat za neoadjuvantnu ili adjuvantu terapiju. Uvođenje neoadjuvantne terapije u liječenje bolesnica s karcinomom dojke stavilo je pred patologa još jednu dodatnu odgovornost a to je da u uzorku tkiva određuje učinak terapije odnosno da drugim riječima ocijeni da li postoji prisutnost tumora nakon terapije ili postoji kompletni patološki odgovor na terapiju kako u primarnom tumoru tako i u zahvaćenom limfnom čvoru metastazama uz prepoznavanje svih morfoloških promjena u tkivu koje stvara citotoksična terapija.

Temeljem svega iznesenoga vidljivo je da se uloga patologa u dijagnostici karcinoma dojke jako promijenila i da je postao ključni član multidisciplinarnog tima za bolesnice oboljele od karcinoma dojke.

Bolesnice s karcinomom jajnika u kontaktu su s brojnim profilima liječnika tijekom svoga liječenja, no vrlo rijetko sa specijalistom koji igra vrlo važnu ulogu kako u postavljanju dijagnoze tako i u njihovom liječenju a to je patolog. Točna patohistološka dijagnoza i točno određeni svi prognostički i prediktvni čimbenici ključ su primjene adekvatne terapije. Granični slučajevi kao i stadij bolesti moraju biti raspravljani u sklopu multidisciplinarnog tima unutar koja je patolog ključna osoba.

Patohistološki nalaz mora sadržavati tip tumora, histološki stupanj diferenciranosti, nalaz mikroinvazije, status limfnih čvorova, peritoneuma, omentuma, jajovoda i stadij bolesti.

Tip tumora određuje se prema klasifikaciji Svjetske zdravstvene organizacije. Epitelni tumori jajnika su heterogena skupina tumora koji se klasificiraju prvenstveno prema vrsti stanica u serozni, mucinozni, endometrioidni, svijetlih stanica, tumore prijelaznih i pločastih stanica. Mnogo je važnije da se svi ti tumori nadalje klasificiraju u podskupine (benigni,granični i zloćudni) ovisno o stupnju proliferacije i staničnoj atipiji kao i prisutnosti ili odsutnosti stromalne invazije. U novije vrijeme, temeljem histopatoloških, imunohistokemijskih kao i molekulsko genetičkih analiza razlikuje se pet glavnih karcinoma jajnika: serozni karcinom visokog stupnja maligniteta (engl HGSC), endometrioidni karcinom (EC), karcinom svijetlih stanica (engl.CCC), mucinozni karcinom i serozni karcinom niskog stupnja maligniteta (engl.LGSC).

Serozni karcinom jajnika je niskog ili visokog stupnja maligniteta i oni su u osnovi različite vrste tumora. Serozni karcinom niskog stupnja maligniteta često je udružen s graničnim karakteristikama, nosioc je KRAS i BRAF mutacija no ne pokazuje mutacije p53 i gena BRCA. Serozni karcinom visokog stupnja maligniteta ima tipične mutacije p53 i nenormalnosti gena BRCA.

Mucinozni karcinom ima stupanj diferenciranosti kao i endometrioidni karcinom maternice, premda se on danas dijeli u dvije podskupine: ekspansilni i infiltrativni. Ekspansilni tip tumora ima bolju prognozu od infiltrativnog tipa.

Endometrioidni karcinom ima tri stupnja (I,II i III) temeljem FIGO klasifikacije koja se upotrebljava za endometrioidni karcinom maternice.

Karcinom svijetlih stanica kao i karcinom prijelaznih stanica automatski imaju visok stupanj diferenciranosti odnosno gradus III.

Mikroinvazija se javlja u slučajevima graničnih tumora obično kod seroznih i mucinoznih tumora, no nema utjecaja na preživljenje a može biti i multifokalna.

Nalaz limfnih čvorova je važan. Mora se naznačiti njihov točan broj, lokalizacija i ukoliko su zahvaćeni metastazama ukupni broj zahvaćenih limfnih čvorova.

Zahvaćenost peritoneuma obično je prisutna u graničnih tumora i svaki uzorak iz te lokalizacije mora biti opisan. Poteškoće su vezane uz točno određivanje da li se radi o pravoj invaziji ili prisutnosti tzv. neinvazivnih implantata što katkada zahtjeva i konzultacije kao i iskusnog patologa u području ginekološke patologije.

Analiza omentuma također mora biti detaljna osobito zbog nalaza neinvazivnih i invazivnih implantata. Ukoliko se nađu invazivni implantati bolesnica je kandidat za adjuvantnu kemoterapiju.

Histološka analiza jajovoda je također vrlo važna, pogotovo kada se danas zna da fimbrijalni dio jajovoda predstavlja mjesto iz kojeg se razvijaju mnogi serozni karcinomi zdjeličnih organa koji u konačnici zahvaćaju jajnike.

Stadij bolesti određuje se temeljem FIGO klasifikacije. Preliminarni stadij može se odrediti na patohistološkom nalazu, no konačni stadij mora biti određen na multidisciplinarnom timu kada se moraju uzeti u obzir svi klinički, radiološki i patohistološki čimbenici.

Katkada se u dijagnostici koristi radiološki vođena biopsija iglom radi postavljanja dijagnoze prije operacije ili kemoterapije. S obzirom da su uzorci mali, takva biopsija zahtjeva visoko kvalificiranog patologa u području ginekološke patologije.

Intraoperativna biopsija na smrznutim rezovima može se iznimno upotrijebiti kada je potrebno na temelju histološke vrste tumora i stupnja diferenciranosti tumora eventualno odrediti liječenje bolesnice i pogotovo ako se radi o mladoj ženi reprodukcijske dobi. No, takva biopsija ima svoja ograničenja i ukoliko patolog ne može dati odgovarajuće odgovore potrebno je napraviti trajne preparate i tek onda odlučiti o daljnjem načinu liječenja.

U novije vrijeme metode imunohistokemije značajno se koriste kod analize tumora jajnika, premda se dobiveni rezultati moraju tumačiti u usporedbi s kliničkim, makroskopskim i mikroskopskim karakteristikama tumora. Korisna je u razlikovanju primarnog od metastatskog karcinoma jajnika (mogući korisni biljezi su citokeratin 7 i 20, CA 125, CEA, CA 19.9, WT 1, TTF-1, estrogenski receptor i CDX 2), nadalje serozni karcinom jajnika daje pozitivnu reakciju jezgara na WT 1, dok su ostali histološki podtipovi negativni kao što su potencijalno korisni biljezi inhibin i kalretinin za tumore spolnih tračaka i stromalne tumore. Epitelni membranski antigen (EMA) i citokeratin 7 pozitivni su u tumori epitelnog podrijetla.

Brojna istraživanja na molekulsko genetičkoj razini u svezi karcinoma jajnika nisu još do sada zaživjela u kliničkoj praksi uz izuzetak geni BRCA 1 i BRCA 2. Premda se karcinom jajnik pretežno javlja sporadično, postoje slučajevi koji su udruženi s mutacijama gena BRCA 1 i BRCA 2. Danas se oni određuju osobito u bolesnica sa seroznim karcinomom jajnika visokog stupnja maligniteta i važan su prognostički i prediktivni čimbenik u njihovom liječenju. Patolog za takvo testiranje odabire najbolji uzorak tumorskog tkiva bez sekundarnih patoloških promjena koji bi eventualno mogli utjecati na rezultate genetskog testiranja a također je potrebno uvijek priložiti i patohistološki nalaz sa svim pokazateljima koji su bitni za tumačenje rezultata molekulskog testiranja.

Zaključno, uloga patologa je vrlo važna u dijagnostici i liječenju karcinoma dojke i jajnika i on je ključna osoba multidisciplinarnog onkološkog tima.

SADAŠNJE MOGUĆNOSTI I PERSPEKTIVE TESTIRANJA SOMATSKIH MUTACIJA BRCA1 I BRCA2 GENA IZ TKIVA TUMORA JAJNIKA

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UVOD: Frekvencija nasljednih mutacija BRCA1 i BRCA 2 gena kreće se u rasponu od 11% do 15,3% (Alsop i sur. 2012). Analizirajući 235 uzoraka tkiva neselektiranih tumora jajnika, Hennessy i sur. (2010) su utvrdili frekvenciju BRCA mutacija od 18,5 %. Analizama uzoraka tkiva tumora i periferne krvi bolesnica, pokazali su prisutnost nasljednih mutacija u 11,5% i somatskih, tumor specifičnih BRCA mutacija u 7% tumora jajnika. Mutacije su puno učestalije u seroznim tumorima jajnika visokog gradusa, osjetljivih na terapiju s platinom u kojima je utvrđena frekvencija mutacija i do 40% (Ledermann et al 2011).

Od studenog 2015. godine u Hrvatskoj je dostupan lijek koji se temelji na inhibiciji PARP enzima (poli(ADP-riboza)polimeraza). Lijek inhibira funkciju popravka jednolančanih lomova DNK. Liječenje je indicirano kao monoterapija održavanja kod bolesnica s recidivom na platinu osjetljivog seroznog epitelnog karcinoma jajnika s BRCA mutacijom (nasljednom ili somatskom).

Brojne BRCA 1/ 2 mutacije mogu nastati na bilo kojem dijelu gena, dokazano ih je više od 4.000 i klasificiraju se na patogene mutacije, varijante nepoznatog kliničkog značenja i benigne polimorfizme.

ISPITANICI I METODE: Dvadeset i jedna bolesnica s dijagnozom epitelnog seroznog raka jajnika visokog gradusa testirana je na prisustvo mutacija u BRCA genima, analizom genomske DNK izolirane iz parafinskih kocki s tkivom tumora jajnika.

Primjenjena metoda masivnog paralelnog sekvenciranja ili NGS (od eng. next generation sequencing) omogućila je sekvenciranje DNK svih eksona (51) i dijelova pobočnih introna BRCA gena, ukupno 16.250 parova baza. Multiplex PCR metodom (167 parova primera) je umnožena kodirajuća genska regija, načinjene su DNK knjižnice i sekvencirane u formatu poluprovodljivih mikročipova s rezultatom očitanja od 250 do 600 tisuća sekvenci po pojedinoj knjižnici. Rezultati su obrađeni bioinformatičkim software-om i uspoređeni sa sekvencama iz nekoliko referentnih BRCA 1/2 baza. (Uređaj PGM ION TORRENT, Ampliseq BRCA1/2 community Panel, v2; Software Ion Reporter Life Technologies, USA).

REZULTATI: Analizom uzoraka genomske DNK iz tkiva tumora jajnika utvrđeno je da 16 tumora sadrži samo benigne mutacije kategorije genomskih polimorfizama. U 5 tumora (23,8%) utvrđene su mutacije u BRCA 1 genu koje pripadaju kategoriji patogenih mutacija (tip 5). Dvije mutacije su locirane u eksonu 11 - p.L1080Ter. i Q563Ter. Obje mutacije sadrže rani stop kodon koji uzrokuje sintezu defektnog protein koji je izgubio C-terminalne regulatorne domene. Dvije mutacije koje su utvrđene u eksonu 21 (p.Q1777fs) i jedna u eksonu 11 (p.P871Lfs) uzrokovale su promjenu okvira čitanja u prijepisu mRNA i sintezu nefunkcionalnog proteina.

ZAKLJUČCI: Visoko osjetljiva i specifična metoda koja se temelji na masivnom paralelnom sekvenciranju omogućuje analizu BRCA mutacija u genomskoj DNK izoliranoj iz parafinskih kocki. Mutacije BRCA1/2 gena koje se kategoriziraju kao patogene, kvalificiraju bolesnicu za usmjerenu terapiju sa inhibitorom PARP enzima.

LIJEČENJE BOLESNICA S RAKOM DOJKE NOSITELJICA MUTACIJA BRCA 1 I 2 GENA

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Rak dojke u nositeljica mutacija BRCA1/2 gena se razlikuje od sporadičnog raka dojke u rizičnosti za obolijevanje od druge maligne bolesti u budućnosti i osjetljivosti na sustavno onkološko liječenje. Kako u današnje vrijeme testiranje za postojanje BRCA1/2 mutaciju postaje dostupno već u času postavljanja dijagnoze raka dojke, BRCA status može biti uključen pri donošenju odluke o preventivnim mjerama i liječenju bolesnica koje su nositeljice mutacija.

Gotovo 70% tumora dojke u nostiteljica mutacija BRCA 1 su trostruko negativni tumori (engl. triple negative breast cancer, TNBC) i pokazuju imunohistokemijske i morfološke osobitosti tzv „basal-like" tumora (engl. basal like breast cancer BLBC). U nositeljica mutacija BRCA 2 gena većina tumora je hormonski ovisno (estrogen receptor pozitivno). Za sada BRCA status u oboljelih od raka dojke ne predstavlja neovisan prognostički čimbenik, ali postojanje mutacije BRCA 1/2 sugerira i ukazuje na osjetljivost i rezistenciju za specifično sustavno liječenje.

Konzervativno lokalno liječenje (poštedni kirurški zahvat i radioterapija) ili mastektomija predstavljaju jednakovrijedne pristupe u lokalnom zbrinjavanju raka dojke u nositeljica mutacija BRCA 1/2. Većina do sada objavljenih radova ukazuje kako se nakon konzervativnog liječenja raka dojke, u premenopauzalnih žena, snižavanjem izloženosti estrogenima primjenom tamoksifena ili ovarijektomijom snižava rizik razvoja ipsilateralnog povrata bolesti kao i novog metakronog ipsilateralnog primarnog raka dojke. Adjuvantna kemoterapije i radioterapija dojke u ove skupine oboljelih također snižavaju pojavnost ipsilateralnog povrata raka dojke. U nositeljica mutacija BRCA1/2 ipsilateralni povrati bolesti se javljaju tipično nakon više od 5 godina od primarne operacije, uglavnom u drugom dijelu dojke i različite su histologije od primarnog tumora što sugerira radije novi primarni tumor dojke nego li pravi lokalni povrat bolesti.

U današnje vrijeme, odluka o sustavnom onkološkom liječenju u bolesnica s rakom dojke nositeljica mutacija BRCA 1/2 gena donosi se na temelju bioloških osobitosti bolesti a ne BRCA statusu. No takav bi se pristup mogao promijeniti budući da novija istraživanja govore za osobit način osjetljivosti i rezistencije na sustavno liječenje raka dojke u nositeljica BRCA mutacija. Gubitak funkcije BRCA 1 i 2 gena vodi k nemogućnosti popravka dvostrukih lomova DNA homolognom rekombinacijom, te se tako može objasniti posljedična nestabilnost genoma i zloćudno biološko ponašanje, kao i izraženija osjetljivost na citotoksične lijekove koji oštećuju DNA i lijekove koji inhibiraju enzim PARP 1 (engl. poly ADP-ribose polymerase 1) koji sudjeluje u popravku jednolančanih lomova DNA.

Sve je više saznanja kako oboljele žene nositeljice BRCA mutacija zbog jednstvene biologije imaju kliničku korist od citotoksičnih lijekova koji oštećuju DNA, kao što su spojevi platine. Znanja se temelje na studijama neoadjuvantnog liječenja kao i liječenja metastatskog raka dojke nositeljica mutacija. Nedavno objavljeni rezultati randomizirane kliničke studije faze III (TNT trial), u bolesnica s metastatskim ili lokalno uznapredovalim recidivirajućim TNBC. U podskupini nositeljica BRCA mutacija postignuti su superiorni rezultati u odgovoru na liječenje s karboplatinom u usporedbi s docetakselom, a u usporedbi s bolesnicama koje nisu nositeljice mutacija kada su liječene s karboplatinom imale su značajno dulje razdoblje do progresije. Mala prospektivna neoadjuvantna studija Byrsog i sur (2009.g.) pokazala je u nositeljica BRCA 1 mutacija odlične rezultate postizanjem kompletnog patološkog odgovora (pCR, engl. pathological complete response) od 80% prilikom primjene cisplatine. Neoadjuvantna studija vođena u MD Anderson, evaluirala je učinak neoadjuvantno primjenjene kemoterapije koja se osnivala na antraciklinima i taksanima u bolesnica s rakom dojke koje su nositeljice BRCA mutacija i onih koje to nisu. Nositeljice mutacija BRCA 1 postigle su statistički značajno bolji pCR (46% vs 22%). Usprkos prethodno navedenim rezultatima, danas još uvijek ne postoji definitivno suglasje i stav o najboljem kemoterapijskom protokolu za oboljele od raka dojke koje su nositeljice mutacije BRCA 1,2. U njih se za sada kod odluke o liječenju preporuča prvenstveno uzeti u obzir standardne prognostičke čimbenike.U

adjuvatnom liječenju je još uvijek standard primjena protokola koji uključuju antracikline i taksane, iako se u nekim kliničkim situacijama može razmotriti primjena spojeva platine.

PARP inhibicija predstavlja obećajvajuću strategiju u sustavnom liječenju oboljelih od raka dojke, nositeljica mutacije BRCA1/2. Trenutno je nekoliko PARP inhibitora (PARPi) uključeno u kliničke studije faze 2 i 3, s ciljem istraživanja sposobnosti inhibicije PARP: olaparib, veliparib, niraparib, talazoparib i rucaparib. Primjenjuju se kao monoterapija ili u kombinaciji s kemoterapijom. Iskustva s olaparibom postoje u liječenju metastatskog raka dojke nostiteljica BRCA mutacija. Olaparib je primjenjivan u bolesnica koje su prethodno primale barem tri linije kemoterapije. Stopa odgovora je bila 54% i 25% (ovisno o dozi lijeka – 400 mg vs 100 mg). U tijeku je nekoliko kliničkih studija, faze III s primjenom olapariba u adjuvantnom (OlimpyA, u žena koje su nositeljice mutacija BRCA 1,2 nakon završenog adjuvantnog liječenja) i u neoadjuvatnom liječenju (Neo-Olympia, M14-011, AFT -04, NSABP B56-1), u kombinaciji s kemoterapijom. Veliparib, PARP1/2i je istraživan u kombinaciji s temozolamidom uz stopu odgovora 37.5% U očekivanju smo rezultata kliničkih istraživanja faze 3 s primjenom PARPi u adjuvantnom, neoadjuvantnom liječenju kao i u terapiji metastatske bolesti. Osim pitanja kliničkog učinka u centru interesa je i potencijalna toksičnost tijekom njihove duže primjene a koja je naročito važna prilikom primjene u adjuvantnom liječenju rane bolesti: rizik za hematološku toksičnost, razvoj novih primarnih tumora. U budućnosti će se također istražiti potrencijalna uloga PARPi u liječenju bolesnica s hormonski ovisnim rakom dojke.

Hormonsko liječenje raka dojke u nositeljica BRCA mutacija, kao i u oboljelih s sporadičnim rakom dojke, ovisno o menopauzalnom statusu, osniva se na primjeni tamoksifena i inhibitora aromataze. Treba napomenuti kako rezultati nekoliko predkliničkih i kliničkih istraživanja sugerira relativnu rezistenciju na tamoksifen bolesnica nositeljica mutacija BRCA 1, međutim ovi rezutati traže daljnju kliničku potvrdu.

TREATMENT OF THE BREAST CANCER IN BRCA1/BRCA 2 MUTATION CARRIERS

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BRCA-mutation associated breast cancer differs from sporadic breast cancer with regard to future cancer risks and sensitivity to systemic therapies. As nowadays genetic testing for BRCA1 and BRCA2 mutations became available at the time of breast cancer diagnosis, BRCA mutation status can be considered when making treatment and prevention decisions for BRCA mutation carriers with breast cancer.

In patients who develop breast cancer with an underlying BRCA mutation, 70% are classified as triple-negative (TNBC) and basal-like (BLBC) on intrinsic expression profiling. Breast cancers associated with BRCA2 mutations are more likely to be estrogen receptor positive. Although at present BRCA mutation status is not an independent prognostic factor, recent research suggests sensitivity and resistance of BRCA mutation-associated breast cancers to specific systemic therapies.

Breast conserving surgery or mastectomy are an adequate local treatment options for BRCA mutation-associated breast cancer. In premenopausal BRCA mutation carriers managed with breast conservation, decreased estrogen exposure achived with tamoxifen or oophorectomy, appear to reduce the risk of ipsilateral recurrence and new metachronous ipsilateral primary breast cancer. Additionally, adjuvant chemotherapy and radiation therapy, also result in reduced risk of future ipsilateral breast cancer events. The ipsilateral recurrences in BRCA mutation carriers typically occur more than 5 years after the primary breast cancer, often in separate quadrants of the breast and have different histology, suggesting that they represent new primary cancers rather than true recurrences.

There is growing evidence that patients with BRCA mutations may have a distinct biology and benefit from platinum compounds both in the metastatic and neoadjuvant setting. The TNT trial prospectively randomized patients with metastatic or recurrent locally advanced TNBC to either carboplatin or docetaxel. The superior response rate for the carboplatin over the taxane was demonstrated in BRCA mutated tumors; and when receiving carboplatin patients with BRCA mutations had superior PFS compared to BRCA-wild-type patients. Exquisitemarkable pathologic complete response rate exceeding 80% has been reported in a small prospective trial evaluating neoadjuvant cisplatin in BRCA1 mutation-associated breast cancer (2009.) Study form MD Anderson that was evaluating response to neoadjuvant systemic therapy for breast cancer in BRCA mutation carriers and noncarriers, demonstrated that BRCA1 carriers had higher pathological complete response to neoadjuvant antracycline-taxane based chemotherapy (46% vs 22%). Still, there is no definitive conclusion on the best chemotherapy regimen for BRCA mutation breast cancer patients. For now, standard progonostic features should be used to decide about treatment. In adjuvant setting chemotherapy should include an antracycline and a taxane. Platinum-based therapy should not be routinely used in the presence of BRCA mutation

PARPs (poly(ADP-ribose)polymerases) are family of enzymes involved involved in DNA-damage repair. PARP inhibition represents promising strategy for the systemic therapy of BRCA mutation-associated breast cancer. Currently, a few compounds with ability to to inhibit activity of PARPs are being investigated in clinical trials: olaparib, veliparib, niraparib, talazoparib i rucaparib. They have been tested as monotherapy or in combination with other cytotoxic compounds. Knowlege and experience with oliparib monotherapy are form metastatic BRCA mutation-associated breast cancer, prevously treated with multiple lines of chemotherapy, with response rate from 25% - 41% (depending on drug dosage – 100 mg vs 400 mg). Several clinical studies phase 3, with oliparib in adjuvant (OlimpyA, in women with BRCA mutation-associated breast cancer after adjuvant therapy) and neoadjuvant setting (Neo-Olympia, M14-011, AFT -04, NSABP B56-1), in combination with chemotherapy are ongoing. Veliparib, PARP1/2i in combination with temozolamid has demonstrated response rate of 37.5% Results of the ongoing phase 3 trials in the metastatic, neoadjuvant and adjuvant setting with PARPi are awaited. Questions neeed to be address are long-term effects and safety during continuous

administration (as is in the adjuvant setting): risk od hematological toxicity or development of new primary malignancies. In the future, the role of the PARPi in the managementof ER positive BRCA mutated tumors will be investigated.

Endocrine therapy in women with BRCA mutation-associated breast cancer as in women with sporadic cancers, includes either tamoxifen or aromatase inhibitors. Some preclinical and clinical dana suggest that BRCA1 mutation associated breast cancer may be resistent to tamoxifen. These results require further clinical confirmation.

PRAĆENJE PACIJENTICA I ZDRAVIH NOSITELJICA MUTACIJA U BRCA1 I BRCA2 GENIMA

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Žene kod kojih su dokazane nasljeđene mutacije u BRCA 1 i 2 genima imaju značajno povećan rizik od obolijevanja od raka dojke i jajnika. Žene nositeljice BRCA 1 i 2 mutacija koje su već oboljele od raka dojke ili jajnika imaju značajno veći rizik da ponovno obole od tih karcinoma, ali i karcinoma nekih drugih organa u tijelu. Postoje dosta velike razlike u zdravstvenoj skrbi i kliničkim preporukama za ovakve pacijentice u različitim zemljama svijeta. Prema preporukama zemalja koje su razvile nacionalne strategije za praćenje i nadzor pacijentica s BRCA 1 i BRCA 2 mutacijama, nositeljice tih mutacija imaju nekoliko mogućnosti koje uključuju pojačane ili redovite preventivne preglede, kemoprevenciju i profilaktičke operativne zahvate. Rano otkrivanje raka dojke za žene s BRCA 1 i BRCA 2 mutacijama uključuje kombinaciju mjesečnog samopregleda počevši od 18. godine života; polugodišnje ili godišnje kliničke preglede dojki od stane liječnika; godišnje mamografske preglede počevši od 30. godine života; i godišnje snimanje dojki magnetskom rezonancom počevši u dobi između 25. i 30. g. života. Pregled dojki magnetskom rezonancom je najsenzitivnija metoda pregleda dojki u nositeljica BRCA 1 i BRCA 2 mutacija i treba biti uključena u svaki program ranog otkrivanja raka dojke. Magnetska rezonanca dojki povećava senzitivnost otkrivanja malignih tumora dojke za oko 81%, dok je senzitivnost mamografije u žena s genetskom predispozicijom svega 40% i ultrazvuka oko 42%. Dodatna dobrobit od magnetske rezonance dojki jest izostanak ionizirajućeg zračenja tijekom snimanja. Trebala bi biti uvedena u program pregleda dojki u dobi od 25 godina ili pet godina ranije od dobi kada je najmlađi krvi srodnik obolio od raka dojke. Godišnji mamografski pregledi trebali bi započeti pet do deset godina ranije nego li je obolio najmlađi krvi srodnik u prvoj liniji krvog srodstva ili u dobi od 30 godina bez obzira kad je obolio prvi krvi srodnik. Međutim, većina smjernica preporučuje redovite mamografije nakon 35. g. života zbog male senzitivnosti ovog načina pregleda u gustom žljezdanom tkivu mladih žena. Ultrazvučne preglede dojki kao neizostavni dio praćenja žena s BRCA 1 i BRCA 2 muutacijama, osobito za mlađe žene s gustim žljezdanim tkivom, preporučuje većina smjernica. Digitalna mamogrfaija i tomosinteza pokazale su se preciznije u odnosu na film-mamografiju u mlađih žena s gustim žljezdanim tkivom. pa se stoga preporučuju za visokorizične mlade žene s BRCA 1 i BRCA 2 mutacijama. Praćenje žena s BRCA mutacijama po pitanju ranog otkrivanja raka jajnika uključuje godišnji ili polugodišnji pregled jajnika transvaginalnim obojenim Dopplerom počevši od dobi od 35 godina ili pet do deset godina ranije nego je obolio najmlađi krvi srodnik u obitelji, te praćenje vrijednosti tumorskog biljega CA 125 u serumu. Nažalost, nema dokaza da redoviti ultrazvučni pregledi jajnika i praćenje CA 125 u krvi smanjuju smrtnost od raka jajnika. Unatoč tome, sve preporuke i dalje uključuju ove metode kao metode rane detekcije raka jajnika u žena s BRCA 1 i BRCA 2 mutacijama, a kojima jajnici nisu odstranjeni. Nadalje, profilaktičke kirurške metode poput mastektomije ili obostrane salpingo-ovarijektomije su metode smanjena rizika od obolijevanja ili metode smanjenja mortaliteta. Utvrđeno je da nositeljice i BRCA 1 i BRCA 2 mutacija postižu dramatično poboljšanje u pogledu smrtnosti obostranim uklanjanjanjem jajnika i jajovodia- za čak 77% do dobi od 70 godina. Ovim postupkom značajno se smanjuje i rizik od razvoja i smrti od raka dojke.- za približno 50%. Profilaktička bilateralna mastektomija povezana je s redukcijom rizika od oko 95%. Međutim, period praćenja žena nakon takvog postupka je kratak za definitivne zaključke. Za već oboljele žene s BRCA 1 i BRCA 2 mutacijama uklanjanje druge dojke i/ili ostatka iste dojke može se preporučiti kao oblik sekundarne prevencije. Za nositeljice mutacija značajan izvor nade jest i razvoj lijekova koji djeluju ciljano na tumorske stanice s oštećenim BRCA i BRCA 2 genima. Jedni od obećavajućih su svakako PARP inhibitori (poli(ADP-riboza)polimeraza1) čije djelovanje su već pokazale kliničke studije. Kemoprevencija za žene s BRCA 1 i 2 mutacijama uključuje selektivne estrogen receptor modulatore (SERM-ove) kao što su tamoksifen i raloksifen te inhibitore aromataze kao što je anastrozol. Tamoksifen u žena starijih od 35 godina može reducirati rizik od invazivnog raka, DCIS (duktalnog karcinoma in situ), te od lobularnih neoplazija. Raloksifen u postmenopauzalnih žena može reducirati samo riik od invazivnog raka dojke. Situacije rizika su definirane u NSABP P1 studiji (1,66% u 5 godina). Inhibitor aromataze anastrozol u postmenopauzalnih žena značajno reducira rizik od raka

jajnika i raka endometrija, kao i od raka kože, kolorektalnog raka, hematoloških malignoma, raka štitnjače i raka urinarnog trakta. Kemoprevencija se može preporučiti samo nakon individualnog i sveobuhvatnog savjetovanja. Stvarna dobit strogo ovisi o procjenjenom statusu rizika, dobi i postojećim čimbenicima rizika ra razvoj nuspojava.

Ključne riječi: nositeljice mutacija BRCA 1 i BRCA 2 gena, magnetska rezonanca dojki, mamografija, ultrazvuk dojki, profilaktička mastektomija, profilaktička salpino-ovarijektomija, kemoprevencija.

SURVEILLANCE OF PATIENTS AND HEALTHY BEARER OF MUTATION BRCA1 AND BRCA2 GENES

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Women who have inherited mutations in the BRCA1 or BRCA2 genes have substantially elevated risks of breast and ovarian cancer.Women with BRCA 1 or BRACA 2 mutations that are suffering from breast or ovarian cancer have a significantly higher risk of re-development of breast or ovarian cancer, or some other organ of the body. There are wide variations in services for these patients and clinical interventions and recommendetions differ between countries of the world. According to the guidelines of the countries that have developed a national strategy for monitoring women with BRCA 1 and BRCA 2 mutations, mutation carriers have various options, including extensive and regular surveillance, chemoprevention and risk-reducing surgery. Early detection of breast cancer for women with BRCA 1 and BRCA 2 mutations includes the combination of monthly breast self-examination beginning at age 18 years; annual or semiannual clinical breast examination by a health care professional; annual mammography beginning at age 30 years; and annual breast magnetic resonance imaging beginning at age 25-30 years. Breast magnetic resonance imaging is the most sensitive diagnostic modality in BRCA 1 and BRCA 2 carriers and should be included in every early detection programme. Breast magnetic imaging has been shown to increase the sensitivity of malignancy detection to about 81%, whereas mammography sensitivity is estimated to be about 40% in women with a genetic predisposition and to breast ultrasound with a detection rate of only 42%. An additional advantage of magnetic breast imaging is the lack of radiation exposure. It should be initiated at age 25 or 5 years prior to the age at wich are youngest affected family member had developed breast cancer. It is generally recommended that annual screening mammography begin 5 to 10 years earlier than the youngest age at which breast cancer was diagnosed in a first-degree relative or at age 30 years, whichever comes first. But, most guidelines recommend annual mammography only in women older than 35 years because of the decreased sensitivity that in observed in the dense breast in young women. Most of the recommendations include ultrasound breast examinations as an indispensable part of the monitoring of women with BRCA 1 and BRCA 2 mutations, especially for young women with dense breast tissue. Digital mammography and tomosynthesis has been shown to be more accurate than film screen mammography in younger women with dense breast tissue for the detection of breast cancer and is recommended for this population at very high risk. Ovarian cancer surveillance for BRCA mutation carriers includes screening with annual or semiannual transvaginal pelvic ultrasonography with Doppler imaging beginning at age 35 years or at 5 to 10 years younger than the age at earliest ovarian cancer diagnosis in the family, along with CA-125 testing.Unfortunately, no evidence shows reduction in ovarian cancer–related death with pelvic ultrasonography and CA-125 screening in these high-risk women. However, these surveillance strategies continue to be recommended for early cancer detection in BRCA mutation carriers who have not yet their ovaries removed. Furthermore, risk-reducing surgeries like mastectomy or bilateral salpingo-oophorectomy are options to decrease the risks of developing cancer and to lower mortality. It found that both BRCA 1 and 2 mutation carriers achieve a dramatic improvement in all-cause mortality by undergoing bilateral salpingo-oophorectomy - a 77% improvement through age 70 years. While much of this reduction in death comes from prevention of ovarian cancer, some is also due to a reduction in the incidence and death from breast cancer- by approximately 50%. Prophylactic bilateral mastectomy is associated with a reduction of breast cancer by 95%. But follow-up period is too short to draw definite conclusion. For affected mutation carriers contralateral and ipsilateral prophylactic mastectomy can be offered as a means od secondary prevention. For mutations carriers hope was raised that targeted chemotherapy will be available in the near future. Preclinical studies with PARP (poly(ADP-ribose)polymerase1) inhibitors showed that BRCA1/2-deficient tumour cells are selectively targeted. Clinical trials are ongoing to prove the clinical applicability and effect. First studies confirm the promising. Chemoprevention for women with BRCA 1 and BRCA 2 mutations includes selective estrogen receptor modulators (SERMs) tamoxifen

and raloxifen and than aromatase inhibitors(AI) anastrozol. Tamoxifen in women older than 35 years can reduce invasive breast cancer, DCIS, and lobular neoplasia. Raloxifen in postmenopausal women can reduce invasive breast cancer only. Risk situation as defined in NSABP P1-trial (1.66% in 5 years). Aromatase inhibitors anastrozole significantly reduce risk in postmenopausal women for ovarian and endometrial cancer, as well as skin, colorectal, hematologic, thyroid and urinary tract cancers. Chemopreventive regimes should only be offered after individual and comprehensive counseling. The net benefit strongly depends on risk status, age and pre-existing risk factors for side effects.

Key words: BRCA 1 and BRACA 2 carriers, breast magnetic resonance, mammography, breast ultrasound, risk-reduction mastectomy, risk-reduction salpingo-oophorectomy, chemoprevention.

PSIHOLOŠKI ASPEKTI GENETSKOG SAVJETOVANJA

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Emocionalno obojane poruke brže se razumiju i dulje pamte stoga se s pacijentima trebamo uskladiti na kognitivnoj i emocionalnoj razini. To je razlog što posljednjih 10 godina, psihološki aspekti genetskog savjetovanja, postaju ravnopravni informativnom dijelu. Cilj im je razumjeti psihološki učinak primanja genetske informacije i genetskih rezultata testiranja za pojedince i obitelji kao i razumjeti čimbenike koji mogu utjecati na donošenje odluka o testiranju. Posebno osjetljivu psihološku skupinu čine zdravi pojedinci koji razmišljaju o prediktivnom testiranju pa je tada važno razmotriti pitanja poput: Pomaže li dobivena informacija osobi da se lakše nosi s genetskim problemom?Jesu li agende savjetnika i savjetovanog usklađene?Je li dovoljno vremena posvećeno psihosocijalnim pitanjima?Važno je provjeriti motivaciju i realističnost očekivanja svakog korisnika genetskog savjetovanja. Što ljudi misle o uzrocima raka, a posebno o genetskim uzrocima raka? Što će dobiti genetskim testiranjem? Potrebno je testirati osobna i obiteljska iskustva s rakom. Generalno većina ljudi misli da se rak događa nekome drugome te da je vjerojatnost obolijevanja od raka vrlo mala, tj. manja nego kod drugih. U obiteljima gdje je učestalost malignih bolesti veća, obično precjenjuju taj rizik. Važno je provjeriti osobna uvjerenja o raku, odkoga je pacijent dobioinformaciju, vidjetipostoje li krivauvjerenja o nasljeđivanju raka te ih korigirati.

Dosadašnja iskustva s bolesti na koju se osoba želi testirati, ima li djece, percepcija vlastitog rizika, percepcija mogućnosti tretmana i prevencije bolesti, percepcija ozbiljnosti bolesti, percepcija vlastitog selfa i tipa privrženost – koliko se sam mogu nositi s tim, otvorenost komunikacije unutar obitelji, sve su to pitanja koja se moraju otvoriti u procesu savjetovanja.

Na genetsko savjetovanje i testiranjese odlučuju osobe s pozitivnom osobnom ili obiteljskom anamnezom, osobe koje traže informaciju kako bi donijeli najbolju odluku za svoje zdravlje ili pojedinci koji žele pomoći drugom članu obitelji u procesu savjetovanja. Financijski razlozi, zabrinutost zbog vlastite reakcije, mogući utjecaj na ostale članove obitelji kao i problemi sa zdravstvenim i životnim osiguranjem, razlozi su zašto neke osobe ne pristaju na testiranje.

Odluči li se pacijent za genetsko testiranje, a u slučaju da rezultat testiranja pokaže da se radi o nositelju BRCA mutacije, tada svakom pacijentu pristupamo individualno poštujući kompleksne komunikacijske zahtjeve priopćavanja takve loše vijesti (svaka vijest koja drastično i negativno mijenja pacijentov pogled na budućnost). Posebno razvijeni protokol u šest korak za saopćenje loše vijesti tzv. SPIKE razvio je Baille 2000. godine.

Tijekom cijelog procesa savjetovanja, mora se poštivati ne-direktivni pristup što znači da savjetnik ne smije utjecati na odluku klijenta/pacijenta. Ona mora biti potpuno autonomna, a slijedi tek nakon što je osoba dobila sve potrebne informacije u emocionalno podržavajućem savjetovanju. Poznavanje psihodinamike omogućit će savjetniku konstruktivni uvid u vlastite nedostatke kako bi jačanjem svojih egosnaga omogućio adekvatnu pažnju i pomoć obiteljima koje savjetuje.U svakom dijelu savjetovanja neophodno je postići atmosferu u kojoj klijent/pacijent može izraziti svoju zabrinutost, postavljati pitanja i reagirati te osjetiti da ga je savjetnik čuo i odgovorio na pravi način.

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INFORMIRANI PRISTANAK

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SAŽETAK

Informirani pristanak ključno je adekvatno i u potpunosti provesti tijekom genetskih testiranja. Pri tome je važan sam proces informiranog pristanka gdje je potrebno što detaljnije i pacijentu razumljivim rječnikom objasniti sve prednosti i nedostatke testa koji će se upotrijebiti .

Potrebno je i pacijenta pitati da li on želi biti informiran o rezultatima testa ili će se informirati nekoga drugog te koje članove obitelji informirati uz dopuštenje pacijenta o rezultatima testiranja. Sam formular za informirani pristanka treba biti pisan razumljivim jezikom prilagođenim pacijentima koji imaju do 10 godina školovanja. Jer u Hrvatskoj prema popisu stanovništva među stanovništvom starijim od 15 godina, njih 30,8 % ima završenu osnovnu školu, 52,6 % srednju školu, a visokoobrazovanih Hrvata je 16,4 %. Ova izlaganja predstavlja formular za informirani pristanka za genetsko testiranje za rak dojke.

INFORMED CONSENT

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ABSTRACT:

It is crucial to properly and fully implement informed consent process in the course of genetic testing. The process itself is important and it is of the essence to explain to the patient in the language that he or she understands and in detail all the advantages and disadvantages of the test that will be used.

It is necessary to ask the patient whether he or she wants to be informed about the test results or he or she would like that someone else want to be informed in his or her place. It is also important to know which family members with the consent of the patient will be informed about the test results. The form for informed consent should be written in understandable language tailored to patients who are under 10 years of education. The reason for this is that in Croatia according to the census among the population older than 15 years of age, 30.8% have completed primary school, 52.6% high school, and 16.4% have higher education. This contribution gives an overview of the form for informed consent for genetic testing for breast cancer.

POSTERS
Posteri

MOLECULAR DIAGNOSTICS OF SPINAL MUSCULAR ATROPHY BY MLPA METHOD IN CROATIAN SUBJECTS

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Background and aim: Spinal muscular atrophy (SMA) is an autosomal recessive disease with an incidence of 4-10 per 100,000 live births. It is caused by mutations in the SMN1 gene of which the absence of exon 7 is the most frequent change in the population. SMA is characterised by progressive muscle weakness resulting from degeneration of lower motor neurons in the spinal cord and the brain stem nuclei. Time of onset of the disease ranges from before birth to young adulthood. The aim of this study was to confirm SMA clinical diagnosis or to detect SMA carrier status by analyzing DNA samples of subjects with SMA symptoms or family history.

Subjects and methods: MLPA method was used to determine copy numbers of SMN1 and SMN2 genes in total number of 486 subjects. Indications for SMA genotyping were: having SMA clinical symptoms, suspection of being a SMA carrier, prenatal diagnosis. Samples were analysed on 3130xl Genetic Analyser capillary electrophoresis instrument by Applied Biosystems.

Results: By analyzing 486 DNA samples, in 38 of them (7.8%) homozygous deletion of exon 7 of SMN1 gene was detected, four of which were prenatal samples. In 45 subjects (9.3%) carrier status was confirmed by detecting only one copy of exon 7 in the SMN1 gene. Two copies of exon 7 in the SMN1 gene were detected in 378 subjects (77.8%) while three SMN1 copies were detected in 25 subjects (5.1%).

Conclusion: Unlike previously used PCR/RFLP method, MLPA detects SMA carriers by determining SMN1 copy numbers. It is also reliable in the context of prenatal SMA testing. The limitation of this method is that it cannot detect other SMN1 point mutations, so clinicaly unambiguous cases which remain unsolved by this testing should be considered for further testing of SMN1 gene by sequencing analysis.

Keywords: spinal muscular atrophy, carriers, gene copy number, SMN1

REPLICATION CHANGES IN B-CELL LYMPHOMAGENESIS

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Over the last decade it has become evident that tumour development and progression are based equally on changes in tumour cells and the interaction of different types of non-tumour and tumour cells that constitute tumour tissue. Communication network of all these cells is based on soluble signalling molecules or cell-to-cell contact, and this complex interchange of information directs tumour development and progression. B-cell lymphomas, as malignant tumours that arise from immune response cells, represent a specific type of tumour tissue because of the high content of other immune cells. Although tumor cells of specific B-cell lymphoma have origin in B-cells from the same differentiation stage, their adaptation to the microenvironment changes equilibrium of proteins involved in replication process and makes them different from each other. Replication process becomes less accurate leading to the accumulation of changes in DNA that contribute to tumor progression. In this study we analyzed expression level of prereplication/replication complex proteins and proteins involved in chromatin reassembly after the replication. Study was conducted on tissue samples with the diagnosis of different B-cell lymphomas and control tonsil tissue. Results showed significant DNMT1, MCM2, CDT1 and p300 overexpression as well as GMNN underexpression during B-cell lymphoma progression. These findings suggest importance of changes in expression levels of genes involved in replication and chromatin reassembly for malignant tumor progression.

MOUSE TERATOMA DEVELOPMENT INHIBITION BY EPIGENETIC AGENTS IN VITRO

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ABSTRACT:

Testicular Germ Cell Tumours (TGCT), although rare, are the most frequent malignancies in young male population and believed to be initiated by epimutations, i.e. aberrant epigenetics, already in utero. Among various, teratoma is the most differentiated TGCT type encompassing all three germ layer derived tissues. Mouse teratoma is a well-established in vitro model which may be obtained by cultivating 7,5–days-old C3H mouse embryos and represent an ideal system to investigate the effect of the most prominent epigenetic drugs and agents.

After embryo isolation, they were treated for two hours with 5-azacytidine, Trichostatin A, Valproat, esiNanog, esiOct3/4 or esiTrrap. Embryos/teratomas treated with esiGFP served as a negative control. The embryos/teratomas were measured on day 0 and for the consequent 7 days of culturing, after which teratomas were scrapped, Sainte-Marie fixed and paraffin embedded for IHC or processed for stemness gene Oct3/4, Sox2 and Nanog expression analysis by ddPCR.

All of the epigenetic drugs and agents significantly reduced teratoma growth, particularly 5-azaC and esiOct3/4 which arrested it entirely. Consistently with growth data, gene expression analysis showed that all drugs and agents inhibited stemness gene expression, especially esiOct3/4 and 5azaC which depleted it completely. IHC analysis of apoptotic activity showed an interesting pattern, but is still under investigation.

This preliminary data notify that epigenetic drugs and agents have a significant inhibitory effect on teratoma growth and ability of reducing stemness in tumour tissue. Indeed, 5azaC and esiOct3/4 pointed at the possibility that TGCT cancer stem cells could be depleted by DNA methylation and RNA interference modulators.

MOLECULAR DIAGNOSTICS OF DUCHENNE-BECKER MUSCULAR DISTROPHY BY MLPA METHOD IN UNIVERSITY HOSPITAL CENTRE ZAGREB

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Introduction: Duchenne-Becker muscular dystrophy (DMD/BMD) is a result of mutations in DMD gene that leads to complete or partial deficiency of dystrophin protein. DMD/BMD is an X-linked disease, males who inherit the pathogenic variant will be affected, and females who inherit the pathogenic variant are carriers and may or may not develop cardiomyopathy. Deletions of one or more exons are detected in 60 - 70% of mutations in DMD, and 80 - 90% in BMD. Duplications are present in 5 - 10% of DMD or BMD.

Methods: Since year 2012 we analyzed 168 DNA samples using MLPA / capillary electrophoresis.

Results: We detected 15 hemizygous deletions of one or more exons, and 2 hemizygous duplications, as well as 6 heterozygous deletion and 3 heterozygous duplications. In 3 families deletion or duplication was inherited from the mother, and in 2 families it was probably de novo deletion, because mother was not the carrier of the mutation.

Conclusion: Due to the large number of exons that can be analyzed in one reaction, MLPA method represents the gold standard for molecular diagnostics of DMD/BMD.

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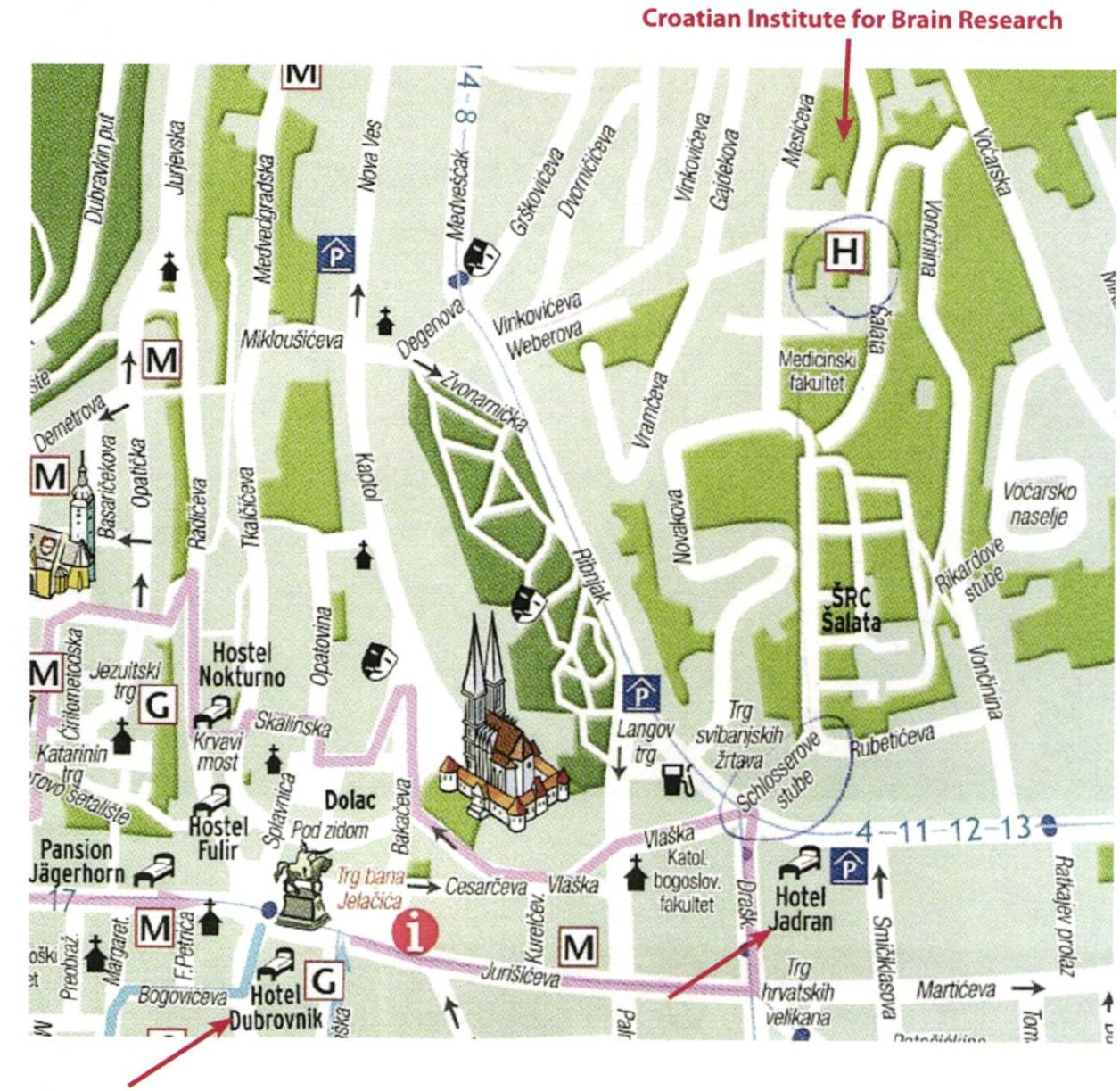
VIEW OF ZAGREB
Pogled na Zagreb

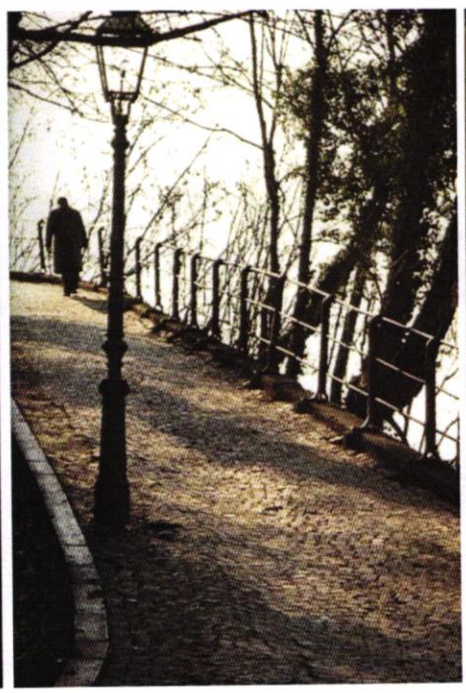
Zagreb



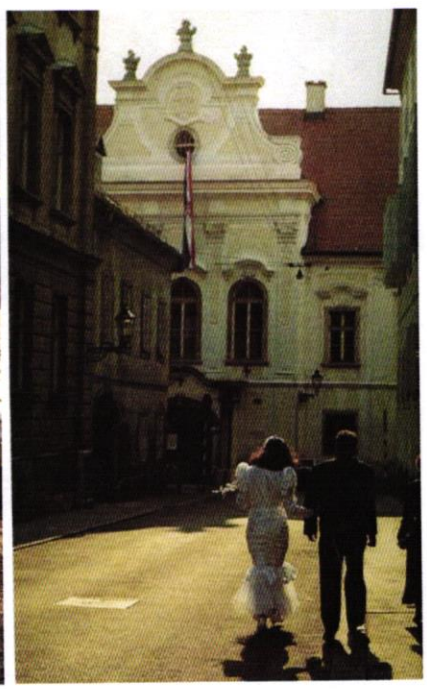
Your hotel "Dubrovnik" at Ban Jelacic Square

Old City Gradec

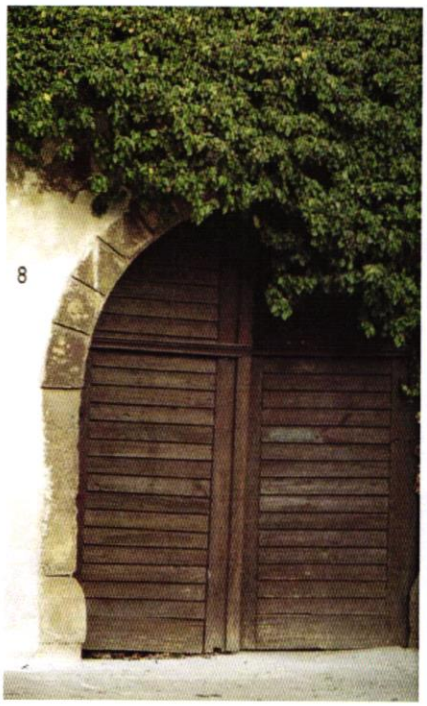
Lotrščak: One of the oldest medieval towers (13th c.)

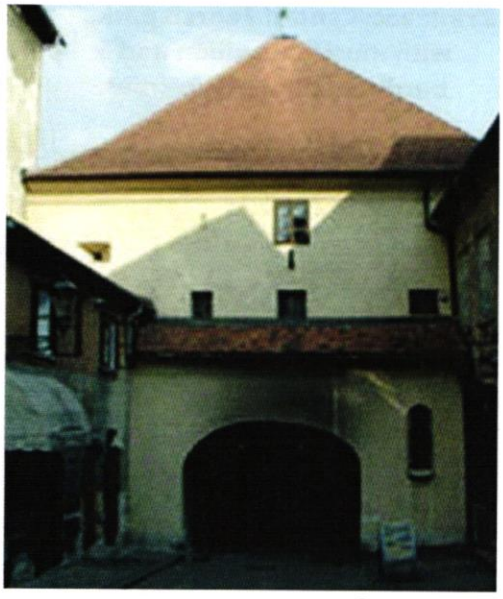


The most popular romantic Upper Town promenade with a view of the city



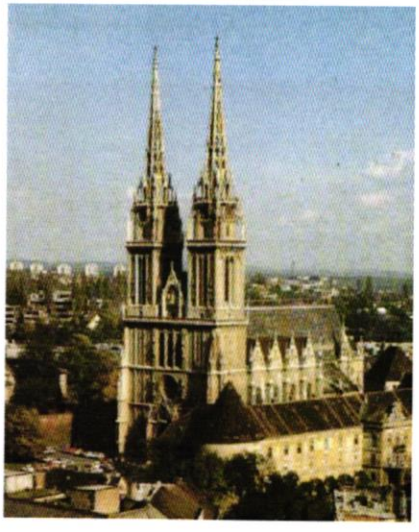
Baroque palace of the Vojković-Oršić-Rauch family n the Matoš Street



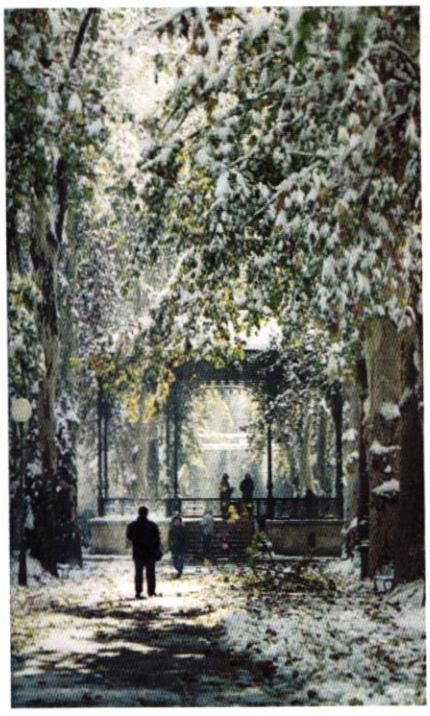


Stone Gate

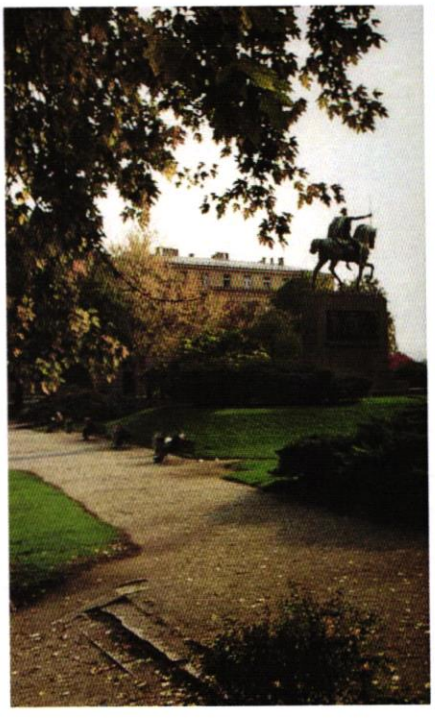
Gradec or Gornji grad



Kaptol and The Zagreb Chatedral



Zrinjevac: Central Zagreb park with musical pavilion and a border of high plane-trees.



King Tomislav Square with park and monument to the first Croatian King

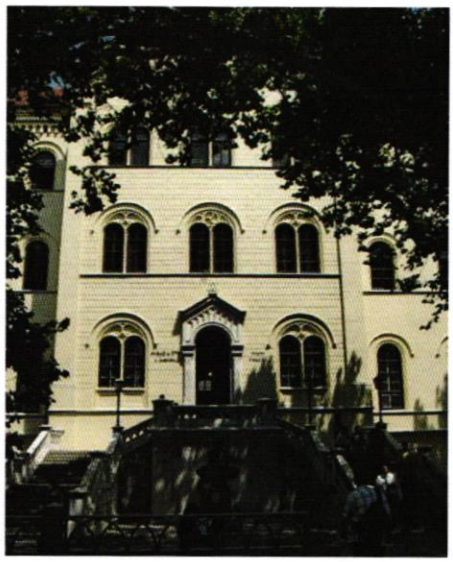
History of Croats. Work of the great sculptor Ivan Meaštrović, exhibited at the Atrium of the Museum on Jesuit Square

Croatian Academy of Science and Arts

Artisticallz wrought iron fence of the palace in Opati;ka Street, built in 1835.

Mimara Museum

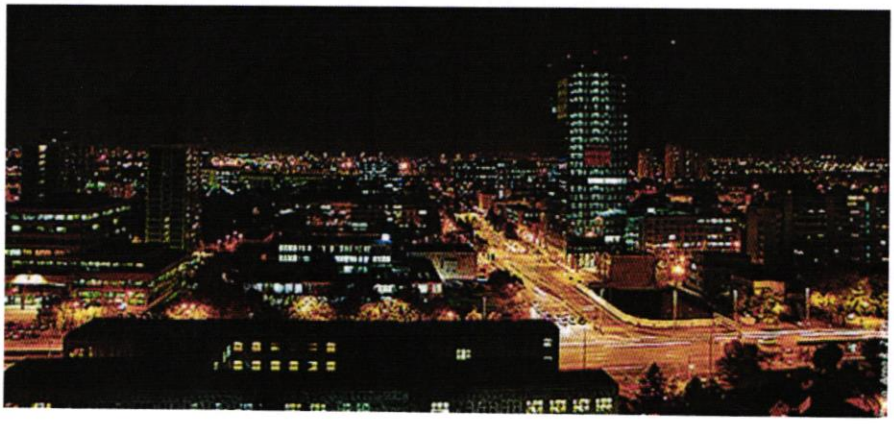
Zagreb Opera House



University of Zagreb
founded in 1669

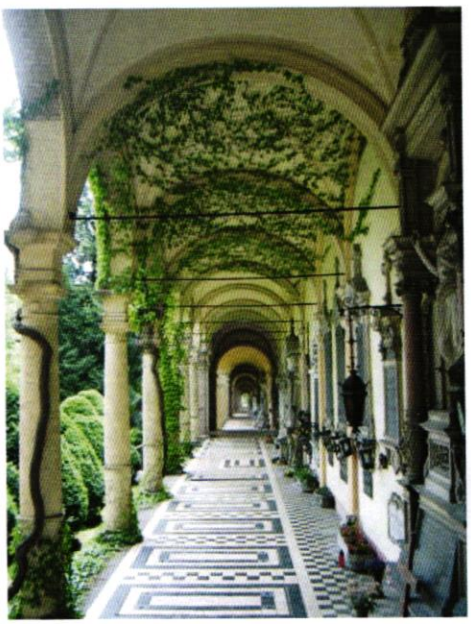
CROATIAN STATE ARCHIVES is one of the most beautiful sites of the art nouveau architecture in Croatia, built in 1913 by the architect Rudolf Lubynski.

Vatroslav Lisinski Concert Hall - Zagreb

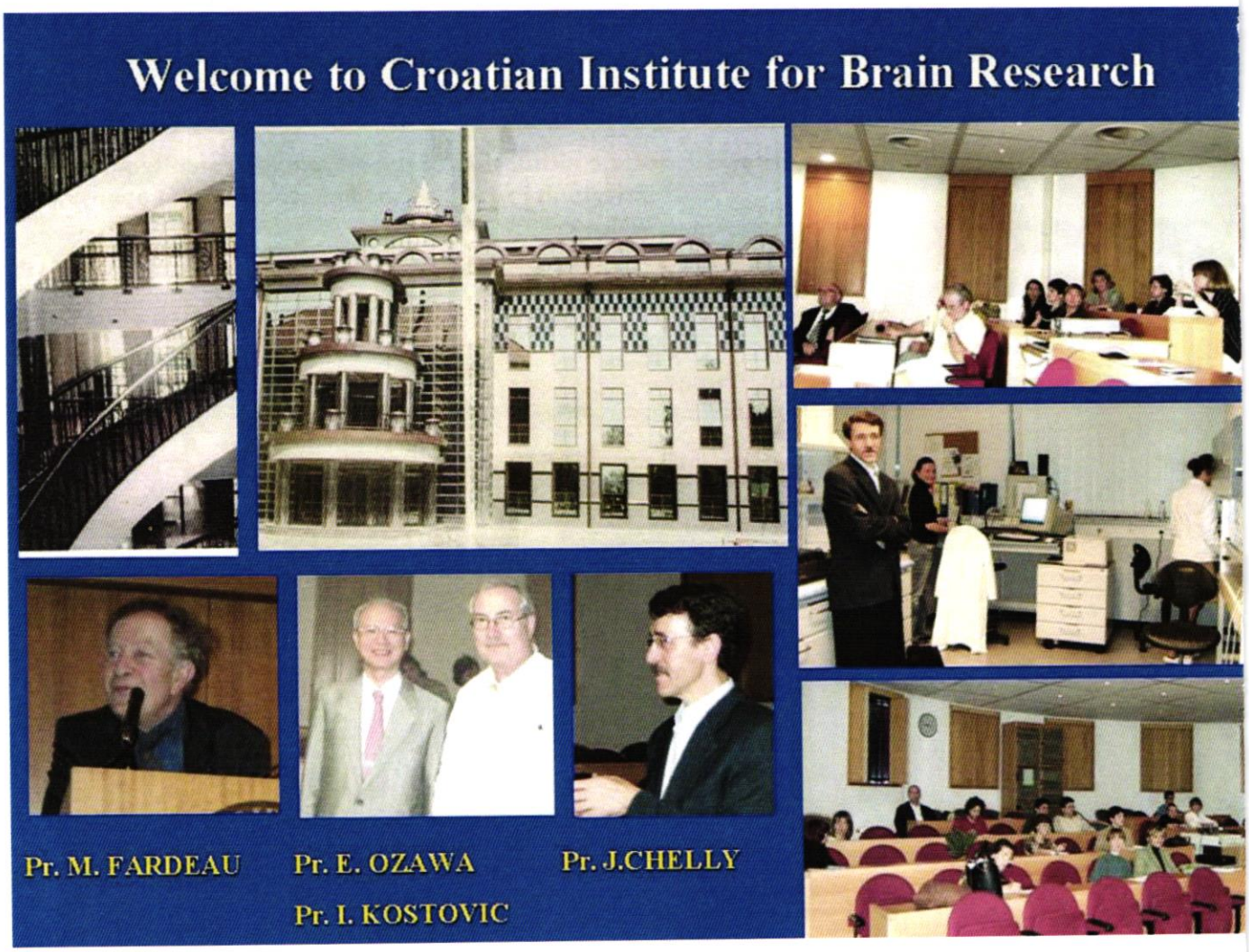


Modern Zagreb at night

Mirogoj Cemetery frontage



Mirogoj Cemetery Arcade in Zagreb



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