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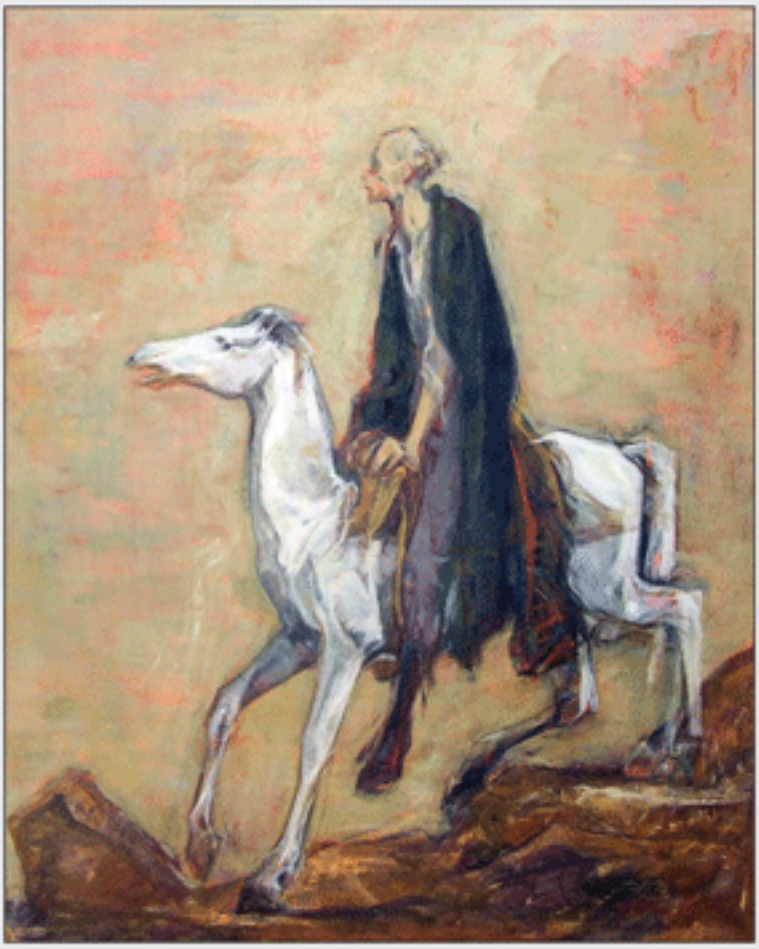

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The Cover Picture: Ismet Mujezinović (1907-1984), *The Typhoid Patient*, Oil on canvas, 162x128 cm. Courtesy of Brčko District Gallery, Bosnia and Herzegovina.

The problems currently faced by biomedical journals in Bosnia and Herzegovina: how to resolve the present situation?

Today's biomedical medical periodicals with scholarly content in Bosnia and Herzegovina number only 10 journals indexed in not very relevant world index bases (apart from the Medical Archives and the Bosnian Journal of Basic Medical Sciences, which are indexed in the Index Medicus/Medline), and they are issued two or four times a year, printed sometimes as double issues, or even quadruple. The reason for this, in the opinion of most editors, is the mediocre quality of the studies sent to the editors of the journals and the lack of funding for publication of journals. These difficulties are characteristic of journals in what is known as the "scientific periphery" (1), and, seen from the perspective of Bosnia and Herzegovina, which is on the periphery of that region, a greater production of scientific works cannot even be expected since the conditions for scientific research in biomedicine are very modest, in terms of the state's science policies, staffing and equipment, since the end of the war to the present. In these conditions, it is almost impossible to plan a long-term strategy for scientific research work for individuals, teams or "scientific institutions" which could realistically make better quality work possible.

For this reason, we are today witnesses of a large number of poor quality studies published in biomedical journals in Bosnia and Herzegovina, which, alongside the difficulties already mentioned, are mainly the result of the laws on institutions of higher education in Bosnia and Herzegovina, which require an unrealistically high number of studies from candidates applying for scientific teaching posts. The insistence on quantity in modest conditions for scientific research work, is stimulation in the wrong direction, that is, stimulation of publication at any price, or, quite honestly, stimulation of the publication of poor quality works. The editors of journals and the review procedure also make a significant contribution to this. That is to say, mainly because of the language in which the studies are written, reviews are written by local reviewers, or reviewers from neighboring countries resulting from the break-up of the former Yugoslavia, that is from an area where almost all professionals know one another, even though it is well known that in these conditions it is impossible to maintain objectivity in the review process (2). Moreover, an analysis of the works published, written by teaching staff at universities in Bosnia and Herzegovina, frequently shows "high scientific productivity" by these staff members in the year before the expiration of their term of office, and the cessation of publication after appointment to a higher position, which all leads to the conclusion that they publish at any cost, and that the legislation on the selection of teaching staff at universities is satisfied in the same way. All this results in

a very meager number of citations of studies published in biomedical journals in Bosnia and Herzegovina, shown by an assessment of their citations in international scientific biomedical journals, which is also confirmed by the fact that no journal from Bosnia and Herzegovina has any official impact factor.

How to resolve this situation?

In this situation the question may quite rightly be asked "Do medical journals in Bosnia and Herzegovina fulfill the basic role of a journal, that is, the transfer of scientific information?" The reply may be found in the fact that the studies published in journals in Bosnia and Herzegovina are very rarely cited in studies by relevant world biomedical journals, meaning that the transfer of scientific information they offer is almost non-existent. It emerges that studies published in journals in Bosnia and Herzegovina only serve the authors to gain promotion on the university teaching profession ladder.

The solution to this situation demands the change of the current, already deeply rooted way of thinking about biomedical studies and journals in both those who decide on the funding that is vital for scientific research work and publication of journals, and those who are involved in scientific research work and publication. This can only be achieved by all those who should be involved in creating a high quality journal taking responsibility for this. Institutions and their founders, who would like their names, whether directly or indirectly, to be associated with scientific activity, much provide financing for scientific research work, and those who do the research and publish it, must accept the international standards for that work. This is not hard to achieve, if there is a common interest involved. These institutions should supply at least 2% of their gross income for scientific research work to meet regulations founded on worldwide criteria for the allocation of those funds, and those doing the research and publishing it, as well as publishing in world journals, should also publish in domestic journals.

The question now arises of which journals in Bosnia and Herzegovina promise progress towards indexation in prestigious world index bases. The answer is clear and unambiguous. These are journals published in English, whose editors strictly respect international standards for the publication of biomedical journals, above all the standards for the review process, that is, those journals which ensure at least three international reviews, of which one must be solely of the statistical processing of data, if statistically tested results are presented. In this process, those who wish to have a good quality work published, do not have to be afraid of the strict supervision of competent reviewers, because this kind of review assesses the study in the true sense of the word, and the useful suggestions are an attempt to improve it, to meet the criteria for publication. This is the way in which we can improve the quality of biomedical journals in Bosnia and Herzegovina, since it should not be forgotten that the value of an article is measured by the value of the journal in which it is published (3).

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Methicillin-resistant *Staphylococcus aureus* (MRSA) in the community – laboratory based study

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Objective To determine the occurrence and antibiotic resistance of community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) isolates. **Methods used** In 2003-2005, consecutive samples of nasal, throat, eye, ear and genitourinary tract swabs, swabs of wound infections and soft and skin tissue infections and samples of sputum obtained from outpatients submitted to the Laboratory with clinical indications were analyzed for the presence of *Staphylococcus aureus*. The disc diffusion method using Mueller-Hinton agar (Oxoid, Besingstoke, UK) was used to test against nine antimicrobials. Oxacillin-resistance was confirmed by E-test (AB Biodisc, Solna, Sweden). **Results** A total of 1583 (11.3%) nonduplicate *S. aureus* isolated from 13 937 samples. MRSA was detected in 63 (4.1%) of S. *aureus* isolates. MRSA isolates more frequently from infected genitourinary tract and wounds than other sites ($p<0.0001$). The patients in both age groups ≥65 and 0-6 years of age were more frequently infected with MRSA than patients of other age groups ($p=0.02$). Statistically significant differences in susceptibility rates between MSSA and MRSA isolates were found for all antibiotic tested ($p=0.0053$ to $p<0.000$). MRSA isolates were more frequently multidrug resistant (MDR) than MSSA isolates ($p=0.0009$). SCC*mec* type IV or V phenotype was detected in 30 (47.6%) of MRSA isolates. **Conclusion** Although low MRSA prevalence was noted, the presence of SCC*mec* type IV/V phenotypes in the community is of particular concern. Effective control of dissemination of MRSA throughout the community will likely require effective control and monitoring of nosocomial MRSA transmission.

Key words: *S. aureus*, MRSA, MSSA, SCC*mec*, Resistance, Multidrug resistance.

Introduction

Methicillin-resistant *S. aureus* (MRSA) has traditionally been considered a hospital-acquired pathogen (HA-MRSA) in patients with established risk factors (recent hospitalization or surgery, dialysis, residence in a long-term care facility, and presence of a permanent indwelling catheter or percutaneous medical device) at the time of culture) (1, 2). But more recently MRSA has emerged as a highly virulent organism in the community of patients without established risk factors for the acquisition of MRSA (3-5). Moreover, the spread of community-acquired methicillin resistant *S. aureus* (CA-MRSA) into hospitals has been reported, causing nosocomial infections (6, 7).

Most studies have been based on hospitalized patients, or patients upon admission to hospital, which has probably resulted in an overestimation of the true prevalence of CA-MRSA (8, 9). Accordingly, epidemiological definitions of CA-MRSA have commonly been based on the timing of isolation of MRSA in relation to the time of admission to hospital, so that MRSA isolates were classified as community-acquired if they were isolated within the first 48-72 h of hospitalization, or if they were isolated in a community setting (10).

Reported prevalence rates of CA-MRSA vary widely among studies, in part because of the use of different definitions used to distinguish between CA-MRSA and HA-MRSA, but also because of the different settings in which studies have been performed. Only a limited number of studies has been performed in outpatient settings and among randomly selected healthy community members (4, 5, 11, 12).

A combination of molecular typing techniques with good resolving power provides a reliable means of analysing isolates of MRSA to determine their genetic relatedness (13, 14). Recent studies have indicated that well-defined CA-MRSA strains carry SCC*mec* type IV or V (14), whereas the majority of HA-MRSA strains carry SCC*mec* type I, II or III (13).

Recently two MRSA strains isolated from the noses and hands of food handlers prompted a retrospective review of Laboratory outpatient records identifying patients from whom *S. aureus* was isolated from any site in the period 2003-2005. The objective of this study was to report the frequency of *S. aureus* isolation in outpatients from the Zenica-Doboj Canton, Bosnia and Herzegovina, according to methicillin resistance, origin of isolates, age and gender of patients, and to determine the antibiotic susceptibility patterns. For comparison, *S. aureus* isolates obtained from food handlers and food products (routinely analysed in the Laboratory during 2003-2004) were also included in the study.

Methods

The Laboratory for Sanitary and Clinical Microbiology of the Cantonal Public Health Institution in Zenica covers a population of 331,229 in the Zenica-Doboj Canton (112,471 males and 218,758 females). In the 2003-2005 period, 13,937 consecutive samples of nasal, throat, eye, ear and genitourinary tract swabs, swabs of wound infections and soft and skin tissue infections (SSTIs) and sputum obtained from outpatients submitted to the Laboratory with clinical indication, were analyzed for the presence of *S. aureus*.

Sterile cotton swabs were used. Swabs were streaked onto sheep blood agar (5% columbia agar base) for detection of gram-positive bacteria, and incubated overnight at 37°C. Morphologically distinct colonies were tested for the production of bound coagulase (Staphylase Test, Oxoid, Basingstoke, UK) and identified as *S. aureus*.

The disc diffusion method using Mueller-Hinton agar (Oxoid, Besingstoke, UK) was used to test against nine antimicrobials (Oxoid, UK). Clinical and Laboratory Standards Institute (CLSI) criteria were used for the interpretation of antibiotic sensitivity testing results (15). Oxacillin-resistant strains were further tested by the E-test (AB Biodisc, Solna, Sweden). Isolates were considered resistant to oxacillin if the MIC exceeded 4 mg/L. The isolates characterized as intermediate by both disk diffusion and E-test were considered susceptible. *Staphylococcus aureus* ATCC 25923 control strains were used. Isolates resistant to oxacillin and susceptible to gentamicin, clindamycin, and trimethoprim-sulfamethoxasole were designated as having a SCC*mec* type IV or V phenotype.

The name, surname, ID, address, gender and age of the patient (0-6, 7-14, 20-64, >64 years), date of isolation, specimen number, source of isolates and susceptibility results of *Staphylococcus aureus* isolates were recorded, as well as the number of specimens submitted during the study.

For comparison, *S. aureus* strains isolated from 4439 successive nasal swabs of food-handlers and 6517 samples of food collected during routine mandatory examination in the Laboratory during 2003-2004 were also included in this study. Microbiological analysis of food products was performed according to the standards and legal regulations of the Republic/Federation of Bosnia and Herzegovina. Routine antimicrobial susceptibility testing of *S. aureus* isolates from these samples was terminated at the end of 2004, and for that reason the data for 2005 were not available.

The significance of differences in resistance rates was determined by means of the χ^2 test and Fisher exact test for independence. A statistically significant difference was defined as a p value of <0.05 and 95% confidence interval.

Results

A total of 1583 (11.3%) nonduplicate *S. aureus* isolates from 13 937 consecutive outpatients presented to the Laboratory because of different clinical symptoms were collected during 2003-2005. MRSA was detected in 63 (4.1%) of S. *aureus* isolates and in 0.6% of submitted samples. *S. aureus* was identified in 322 out of 4439 (7.3%) nasal swabs of food handlers, five of which were MRSA (1.6%). MRSA was isolated in 0.1% of submitted food handler samples. Thirty five *S. aureus* strains were isolated from 6517 (0.5%) food samples, and two of them (5.7%) were MRSA. All *S. aureus* isolated from ice cream samples obtained from local patisseries and fast food restaurants.

Table 1 shows the distribution of methicillin susceptible *S. aureus* (MSSA) and MRSA isolates according to the origin of isolates.

MRSA isolates were more frequently isolated from genitourinary tract and wounds than from other sites (p<0.0001).

The patients in age groups ≥65 and 0-6 years of age were more frequently infected with MRSA than patients of other age groups (p=0.02) (Table 2). Female patients were significantly more often infected with MRSA than male patients (p=0.003) (data not shown). The median age of patients infected with MRSA and MSSA was 30.09 and 20.88, respectively.

Statistically significant differences in susceptibility rates between MSSA and MRSA clinical isolates were found for all antibiotic tested (p=0.0053 to p<0.0001) (Table 3). No resistance to vancomycin or ciprofloxacin was detected in MRSA isolates. MRSA isolates were more frequently multidrug resistant (MDR) than MSSA isolates (p=0.0009). According to origin, MDR was more often detected in wound infection isolates, 28.6%, than in isolates from GU tract and nose, 12.5% and 0.6%, respectively, but with no statistically significant difference (data not

Table 1 Distribution of MRSA and MSSA clinical isolates of different origin in the 2003-2005 period

Origin of isolates	Site of isolation	No of samples submitted	No of MSSA	No of MRSA (% of SA)	No of MRSA with SCCmec IV or V phenotype	Total S. aureus (% of submitted samples)
Clinical	Nos	7978	1146	34 (2.9)	21 (61.2)	1180 (14.8)
	Throat	12.032	10	1 (9.1)	0	11 (0.09)
	Sputum	14	2	0	0	2 (14.3)
	Wound	444	168	14 (7.7)	5 (35.7)	182 (41.0)
	SSTI	217	4	0	0	4 (1.8)
	Eye	1808	106	5 (4.5)	3 (60)	111 (6.1)
	Ear	379	45	1 (2.2)	0	46 (12.1)
	Genito-urinary tract	1065	39	8 (17.0)	1 (12.5)	47 (4.4)
	Total clinical	13937	1520	63 (4.0)	30 (47.6%)	1583 (11.3%)
Food handlers	Nose	4439	317	5 (1.6)	5 (100)	322 (7.3)
Food	Food samples	6517	33	2 (5.7)	2 (100)	35 (0.5)

Table 2 Distribution of MRSA and MSSA clinical isolates according to age groups

	Age groups				
	0-6	7-14	15-19	20-64	≥ 65
	Number (%) of patients				
MRSA	11 (20%)	10 (18.2%)	4 (7.3%)	23 (41.8%)	7 (12.7%)
MSSA	441 (32.4%)	331 (24.3%)	107 (7.9%)	415 (30.5)	68 (5.0%)
Total	452 (31.9%)	341 (24.1%)	111 (7.8%)	438 (30.9%)	75 (5.3%)

Table 3 Antimicrobial resistance patterns of MSSA and MRSA isolates in the 2003-2005 of different origin

	Origin of isolates	S	R	MDR	Percentage of resistance to antimicrobial agents*								
					VAN	GEN	KAN	ERY	TET	CIP	CLI	SXT	CHL
MSSA													
	clinical (1520)	1091 (71.8%)	429 (28.2)	23 (1.5)	0	5.3	8.7	7.1	17.1	0.6	1.6	4.4	2.7
	food (33)		8 (24.2)	0	0	3.6	6.1	6.1	28.1	0	0	0	0
	food handlers (317)		64 (20.2)	0	0	0.7	3.3	5.9	14.6	0.7	0	2.6	2.6
MRSA													
	clinical (63)	16 (25.4%)	47 (74.6)	10 (15.9)	0	17.9	36.8	37.1	31.7	0	23.0	31.7	9.8
	food (2)		2	0	0	0	0	100.0	50.0	0	0	0	0
	food handlers (5)		3	0	0	0	25.0	40.0	40.0	0	0	0	0

MSSA, methicillin-sensitive *Staphylococcus aureus*; MRSA, methicillin-resistant *Staphylococcus aureus*; S, susceptible; R, resistance to one or more antimicrobials; MDR (multidrug resistance), resistance to three or more antimicrobials
*Antimicrobial agents tested: vancomycin (VAN), gentamicin (GEN), kanamycin (KAN), erythromycin (ERY), tetracycline (TET), ciprofloxacin (CIP), clindamycin (CLI), trimethoprim-sulfamethoxasole (SXT), chloramphenicol (CHL)

shown). No MDR was detected in MSSA and MRSA isolated from food handlers or food products.

SCC*mec* type IV or V phenotype (isolates resistant to oxacillin and susceptible to gentamicin, clindamycin, and trimethoprim-sulfamethoxasole) was detected in 30 (47.6%) of MRSA isolates. These MRSA phenotypes were significantly more often isolated from GU tract, wounds and nose than from eyes (p=0.0005), but they were not isolated from throat, sputum or ear (Table 1).

Discussion

The finding of 30 MRSA isolates showing good sensitivity to antibiotics other than beta-lactams and the low prevalence of multidrug resistance (MDR) in MRSA suggests the presence of true CA-MRSA in our population (2-4, 16) Multidrug resistance characterizes nosocomially acquired MRSA strains isolated from patients with identified risk (2,4).

Nasal carriage of *S. aureus* is an important risk factor for infections by this organism in both community and hospital settings (16). Health-care exposure is significantly associated with MRSA carriage (10, 18). In our study MRSA was detected in 0.6% of clinical samples submitted to our Laboratory, which is in agreement with colonization reported among community members without healthcare contacts in the USA (0.2%) and Europe (0.7%) (10, 19).

It has been documented that CA-MRSA infections have been increasing among adults and children (4, 20). The results of the present study have also shown that MRSA more often infected the oldest (≥65) and youngest (0-6) age groups of patients than other age groups. Therefore, microbiologic culture and antimicrobial susceptibility testing would be recommended to guide treatment.

The prevalence of colonization of both *S. aureus* and MRSA in food handlers and their appearance in food products was low and in agreement with the prevalence of *S. aureus* and MRSA infections in our region. Reportedly, MRSA-contaminated food can be a vehicle of outbreaks affecting low-risk persons within the community and the food was contaminated by an asymptomatic carrier (21). There were no *S. aureus* foodborne outbreaks noted in this period.

The spectrum of illness is similar for MRSA and MSSA infections in our community, but we found that MRSA were more often isolated from the GU tract and wound infections than from other sites.

Susceptibility results for MRSA demonstrated that the prevalence of resistance to ciprofloxacin and erythromycin was as high as 80% and 90%, respectively (22, 23). Fluoroquinolone resistance emerged very rapidly in HA-MRSA in the years after widespread utilization of these agents (23-25). No resistance to fluoroquinolones was noted in this study in MRSA isolates of any origin investigated, but interestingly, it was detected in MSSA isolated from clinical samples and food products.

We found 47.6% MRSA isolates having the SCC*mec* type IV / V phenotype, which is typical for CA-MRSA isolates (7). All MRSA isolated from food handlers and food products (ice cream) were SCC*mec* type IV or V phenotype. SCC*mec* type IV/V type has increased mobility and therefore greater potential for horizontal spread to diverse *S. aureus* genetic backgrounds, compared with other SCC*mec* types (13, 14). We did not perform genotype confirmation of SCCmec type IV or V phenotype, but according to the high correlation between the genotype and phenotype we could assume that at least some of these MRSA strains are generated in the community.

Our investigation has some limitations. This is a retrospective study with a relatively small sample size and accordingly, a small number of MRSA were analysed. Additionally, molecular analysis was not perfomed and a risk factors involved in acquisition of MRSA infections wre not investigated. Also, data on the prevalence of HA-MRSA in this region are missing. But, since we found that 25.4% (16/63) MRSA isolates were fully susceptible to all antibiotic tested and 30 (47.6%) MRSA isolates had SCC*mec* IV/V phenotype we could estimate that MRSA generated in the communitya might be present in this region.

The origin of CA-MRSA strains is still the subject of debate**.** Only studies based on appropriate molecular analysis would be able

to determine these newly identified community-acquired strains. Further population-based studies in outpatient settings are warranted in order to define fully the extent of MRSA infections without identified risk.

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Hereditary non polyposis colorectal cancer in a random sample of colorectal cancer patients

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Objective The goal of this prospective research was to determine genetic and endoscopic changes in patients with sporadic colorectal cancer and to diagnose HNPCC. **Patients and Methods** The group consisted of 40 patients, having colorectal cancer. Colonoscopy was performed, genetic testing for the loss of heterozygousity and microsatellite instability (MSI). **Results** HNPCC was diagnosed using the Amsterdam and Bethesda criteria in the group of sporadic colorectal cancer in 15% of the cases, and exhibited an MSI-H for the chromosome 2p where the hMSH2 mismatch repair gene is localized. The greatest number of patients with sporadic colon cancer and HNPCC displayed Astler-Coller B2 and C1 spread levels. **Conclusion** The research results indicate that the colonoscopy should be used as a screening method for colorectal cancer. It is necessary to design a colorectal cancer screening program for the general population and high risk individuals. There is a need to form National colorectal cancer, HNPCC and FAP registries.

Key Words: Colorectal cancer, Genetics, Endoscopy, HNPCC.

Introduction

Colorectal cancer is one of the most frequent: it is the second most frequent in men and women, just after lung cancer and breast cancer, respectively. A significant number of people are at high risk of developing colorectal cancer if they carry germ line mutations. Molecular genetics promises to be the key for identification of the population at high risk of developing colorectal cancer. Genetic testing could be clinically used in the future to diagnose sporadic and hereditary colorectal cancer.

Mutations cause uncontrolled multiplication and the uncontrolled growth of mutated cells within the body. Mutations of control genes (control the cell cycle) cause genome instability. The most frequent mutation is of the p53 gene – a guardian of the

genome. p53 protects DNA by blocking cell proliferation, stimulating DNA repair and promoting apoptosis. Neoplastic growth is inevitable without apoptosis (1).

All cancers are genetically caused. Development of cancer is the result of mutations in tumor suppressor genes, activation of oncogenes and mutations in DNA repair genes (MMR). Genetic pathways of cancer pathogenesis (2) are: the loss of heterozygosity (LOH) and defects in mismatch repair (MMR) genes.

Loss of heterozygosity is the complete loss of function of two corresponding genes (alleles)-deletion. The most frequent areas are 17p and 18 where tumor suppressor genes are located.

Defects in mismatch repair (MMR) genes are changes in short sequences of bases that are repeated and are found in the entire genome of cancer DNA, which is damaged in microsatellite regions-microsatellite instability (MSI). This is formation of new alleles at microsatellite loci in tumor DNA.

Hereditary cancers are microsatellite instability (MSI) in 90% of cases, and 10% of sporadic cancers are MSI. MSI causes an expression of transformed tumor growth factor on the cell surface (3).

According to the hereditary influence, cancers are classified as: sporadic colorectal cancer (60%), familial (30%) and hereditary (10%). There is average, increased and high risk for development of colorectal cancer.

Hereditary nonpolyposis colorectal cancer (HNPCC) is a condition caused by an alteration in one or more of the MMR genes. In around 50% of cases there is a chance of passing the causative gene to children. Carriers of genetic mutations have an 80% chance of developing cancer. This condition should be suspected when the Amsterdam II or the Bethesda-modified criteria are present (4).

Amsterdam criteria were established for diagnosing HNPCC (5); there should be at least three relatives with an HNPCC – associated cancer (colorectal cancer, cancer of the endometrium or ovarium, stomach, small bowel, pancreas, biliary tract, ureter or renal pelvis and brain). All the following criteria should be present: one should be a first degree relative of the other two; at least two successive generations should be affected; at least one cancer should be diagnosed before age 50; FAP should be excluded in the colorectal cancer case (if present); tumors should be verified by pathological.

Bethesda Guidelines were developed in 1996 and revised in 2000, in which criteria for the identification of colorectal tumors that should be tested for MSI were present (6): those who meet the Amsterdam criteria should be tested for MSI.

Cancers which are MSI should be tested by sequencing on germ line mutations. Genetic testing using gene sequencing of at least MSH2 and MLH1 that account for approximately 70% of HNPCC cases is the most sensitive and should be considered for affected individuals in families meeting the criteria and first-degree adult relatives of those with known mutations.

The latest genetic information, in future could be clinically significant in the prevention of colorectal cancer, pre-symptomatic cancer diagnosis, selection of patients for the most suitable treatment and evaluation of the malign potential (7).

Genetic therapy will probably be used in the future as the most optimal type of the therapeutic treatment.

There is no colorectal cancer registry in Bosnia and Herzegovina, but there is registry in the Tuzla county with 500,000 inhabitants. Until now colorectal cancer has only been diagnosed as sporadic. The aim of this research was to select individuals and families who should be screened for genetic testing for HNPCC and to diagnose hereditary colorectal cancer.

Material and Methods

Material

This prospective study was undertaken in the period from January to December 2000, in the Clinic of Internal Diseases, Surgery Clinic, and Department of Pathology in University clinical center Tuzla, Institute for Biochemistry, Medical School of Ljubljana, and Institute of Biochemistry, University of Oxford. The sample involved 40 patients with sporadic colorectal cancer. During this year in the Endoscopy Unit 152 colorectal cancers were colonoscopicaly diagnosed and 1200 colonoscopy were performed. In this sample there were 7.8% colorectal cancers found in the total of 1200 colonoscopy procedures. The sample size was limited to 40 colorectal cancers due to lack of resources, however we believe that this size is sufficient for the benefit of this study. The first page of the registration chart was reserved for personal data and an accurate genealogical tree, in which the main causes of morbidity and mortality of first degree relatives were recorded. Familial disease history analysis gave the data on colorectal cancer presence, adenoma, or other cancer in the family. The Amsterdam Criteria and Bethesda Criteria were used for genetic tests and HNPCC was diagnosed in the patients with previously diagnosed sporadic colorectal cancer.

Sporadic cancer is characterized by genetic mutations existing just in the cancer cell populations. In hereditary colorectal cancer genetic mutations exist in the cancer cell population, in surrounding tissue and peripheral blood. So, in this group of patients the control group are cells of surrounding healthy tissue.

Genetic tests of cancer tissue were undertaken and surrounding tissue was tested. The kind and frequency of genetic mutations characteristic for our tested group were identified.

Linkage analysis was performed to identify the LOH, as well as homozygosity for tumor suppressor genes. Genetic tests for microsatellite instability mismatch repair gene with identical microsatellite markers were carried out. In this way the most effective molecular screening for HNPCC was identified.

Methods

Genetic tests were performed by molecular biology methods. DNA isolation was performed from cancer tissue and the surrounding healthy tissue.

Genomic DNA isolation was performed on deparafinization tissue sections as on cell proteolises of tissue by proteinase K and was checked by spectrophotometer and it ranged from 100-200 ng per sample (8).

Polymerase chain reaction (PCR): Chain DNA synthesis reaction by means of polymerase (PCR) is a method by which a specific DNA cut may be multiplied into a huge number of copies. A mixture of genome DNA with the gene that we want to multiply, individual nucleotides: a pair of initial oligonucleotides, Taq polymerase and Mg+ with optimal ph, should be made for PCR reaction. The PCR conditions were as follows: after initial 2 min denaturation at 94° C, 30 amplification cycles were performed, each consisting of 10 s steps at 94°C, 30 s steps at 55° C, and a 30 s elongation step at 72° C. Amplification was completed with a final incubation step at 72°C for 7 min. For separating of amplified PCR products, we used an automatic sequencer 310 AB1 PRISM, Genetic Analyzer 310 (Perkin Elmer), which enable separating and quantification of DNK fragments according to the principle of capillary electrophoresis. PCR was performed using a PCR Thermocycler 9600 (Perkin-Elmer).

Fluorescent PCR is the method of wide usage in tumor detection, particularly im-

portant in MSI determination, and determination of LOH in tumor suppressor genes. Multiplied products with fluorescently marked one chain of DNA molecules were obtained.

Fluorescent PCR of microsatellite markers: mononucleotides and dinucleotides markers were used for detection of microsatellite instability on MMR genes. In the group of mononucleotides markers BAT 25 were used (for microsatellite locuses on chromosome 4q12), BAT 26 (chromosome 2), and BAT 40 (chromosome 1P13.1). In the group of dinucleotide markers DS 123 were used (chromosome 2p16), and TP 53 (chromosome 17p13.1). Initial nucleotides in the marker BAT 25 were marked with HEX fluorescent dye. Predenaturation was done for each locus at 90^0C, the temperature of annealing of primers and cycles number, the reaction of extensions of oligonucleotides. Conditions for amplification in a Master mix final volume of 25μl was: 2,5 μl 10 % puffer; 1μl DNA (5-10 ng); 2 μl (25 Mm) mGcl2; per 0,5 μl (2,5 mM) of each dNTP-a; 0,1 μl (0,5U) AmpliTaq Gold polymerase (Perkin-Elmer); 1 μl forward primer (12,5 pmol) and 1 μl reverse primer (12,5 pmol) by each pair of initial oligonucleotide for each microsatellite marker, and until 25 μl volumes of sterile water

Multiplex PCR: it is used for more prompt detection of loss of heterozygosity (LOH) of tumor suppressor genes. For Fluorescent detection LOH intragenic markers for the following tumor suppressor genes were used: NM 23 (chromosome 17p 21.3), p 53 (chromosome 17p 13.1), chromosome 5q 21-21), RB (chromosome 13q 14.1-q14.2), DCC1 and DCC2 with HEX. Initial oligonucleotides markers were marked by fluorescent dye for APC and RB1 with TET, markers for NM 23, p 53 and DCC1 with 6-FAM and DCC2 with HEX. Initial oligonucleotides of the chosen markers were multiplied for the locuses: NM 23, p 53, APC, RB1, and in the second, so called duplex reaction, initial oligonucleotides were multiplied for the locuses DCC1 and DCC2. The quality of multiplication and presence of possible contamination of PCR products was checked on 2% agar-gel.

Capillary electrophoresis on automatic sequenator: for separation of amplified products automatic sequenator 310 Genetic Analyzer was used. It enables fragmentation and quantification of DNK fragments by the principal of capillary electrophoresis. For microsatellite analysis and analysis of loss of heterozygosity we used fluorochromes: 6-FAM, TET, HEX (that marked 5 end) and GS TAMRA standards. Each color reflects the strongest fluorescence on different wavelengths. Gene Scan Tamra 500 was used for the analysis of the fragment's size ranging from 35 to 500 bp.

Microsatellite analysis: fluorescent peaks are detected by means of software and shown on anelectrophoregram. Each fluorescent peak is automatically quantified according to the size of basis, height, and in the peak field. All the tests were repeated twice in order to confirm the obtained results.

RER analysis: each sample that had a new allele appearance in the tumor tissue in comparison to the corresponding normal tissue of the sample, represents microsatellite instability for the given marker.

LOH analysis: heterozygosity loss was calculated by mathematics (9). For heterozygosity cases the ratio of allele was calculated for each pair of normal and tumor tissue by the formula: T1:T2/N1:N2, where T1 and N1 values of the fields of shorter allele length, and T2 and N2 fields of longer allele fields for tumorous and healthy samples. In the cases where the allele ratio was above 1.00 the conversion 1 (T1:T2/N1:N2) was used. The result ranged from 0.00-1.00. If the result was under 0.50, significant heterozygosity loss of the longer allele was shown. Homozygous locuses were not taken for the analysis of LOH.

Colonoscopy is an endoscopic procedure made by videocolonoscope Olympus and used for diagnosing colorectal cancers, and for taking a sample for pathohistological analysis. For each of the patients three samples were taken from the cancer and two from healthy tissue 5 cm away from the cancer (control).

Results

Forty patients with sporadic colorectal cancers were diagnosed using endoscopic, surgical and pathohistologically methods. Family history indicated that 7 patients had family members with colorectal cancer or other HNPCC related cancers that were younger than 50 years of age. Amsterdam criteria were used to study these patients, and a genealogical family tree was drawn up for each patient.

By analyzing data according to the Amsterdam criteria, these families were classed as belonging to the HNPCC families. Two of them had family members with colorectal cancer, and four with colorectal as well as other HNPCC cancers. The other patients with colorectal cancers were classified as sporadic colorectal cancers as they did not satisfy the Amsterdam criteria.

During the analysis of age and sex of the 7 patients, it was found that three belong to the 31-40 age group, and the remaining four to the 41-50 group. There were six male and one female patient.

In this study we had 6 HNPCC families, and among the examined patients two of them were related (brother and sister). Therefore, 15% of the patients belong to HNPCC families and the rest to the sporadic colorectal cancers.

Loss of heterozygosity (LOH) in APC gene is present in 84.2% of patients, whereas, LOH in DCC1 and DCC2 is present in 42.8% of the patients. Homozygosity in DCC1 was found to be present in 42.8% of the patients (Table 1).

Loss of heterozygosity also referred to as allele imbalance (AI) and homozygosity (H) are indicated.

Two out of seven patients showed microsatellite instability on 4 markers (MSI-H), 2 on 3 and 3 on 2 markers (MSI-H). MSI on BAT40 is present in 84%, on DS123 in 71.4%, on TP53 in 57.1%, on BAT25 and BAT26 in 42.8% of patients (Table 2).

Microsatellite instability is shown in figure 1.

Cancers were diagnosed colonoscopically in the rectum region in three patients, in the ascending colon region in two patients, hepatic flexure in one patient and colon sigmoideum in one patient.

Adenocarcinoma was diagnosed pathohistologically in 5 patients, and adenocarcinoma mucinosum in 2 patients. According to the Astler-Coller classification of colorectal cancer, three of the patients are classified as C1 and four as C2.

Table 1 Genetic changes in tumor suppressor genes

No. of patients	Genetic changes	Tumor suppressor genes					
		NM23	p53	APC	RB1	DCC1	DCC2
7	AI	2	1	6	1	3	3
7	H	0	0	0	0	3	2

Table 2 Microsatellite instability in patients with HNPCC syndrome

No. of patients	Microsatellite instability				
	BAT25	BAT26	BAT40	TP53	DS123
7	3	3	6	4	5

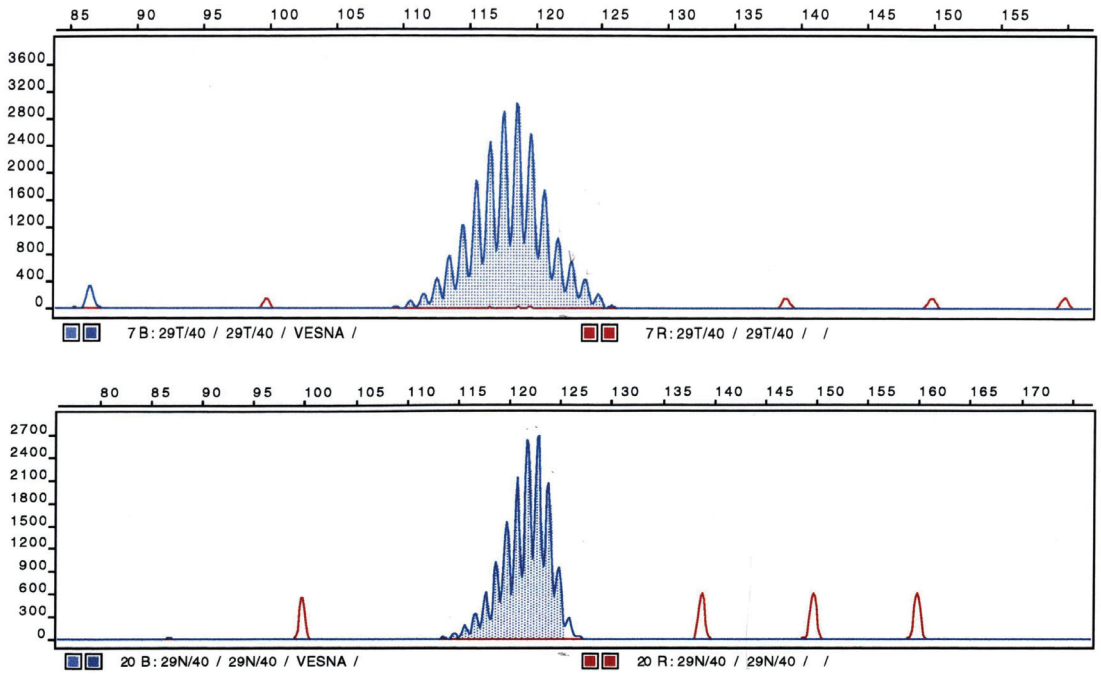

Figure 1 Electropherogram-microsatellite instability in BAT40 (tumorous and healthy tissue)

Discussion

In Bosnia and Herzegovina there has been no study regarding the hereditary forms of colorectal cancer nor is there a national cancer registry. In the Tuzla County in the year 2000, there were a total of 152 patients with colorectal cancer. The incidence rate for men is 6.64 per 100,000 inhabitants and for women 7.42 per 100,000 inhabitants.

In our study, HNPCC was diagnosed in 15% of the patients with colorectal cancer. It is necessary to mention that this research did not cover all patients with the diagnosed colorectal cancer in 2000.

Ponz et al. (10) diagnosed 47 HNPCC-s in 28 families from 1831 cases of colorectal cancers, which was 16% of the total number of patients. Aoltonen et al. (11) gave different results. From 509 tested patients with colorectal cancer only 2% belonged to HNPCC.

We compared our results with both groups of researchers and found that, beside the group of HNPCC they alsohad a group of ones with suspected HNPCC. That wasthe group of older patients who meet the Amsterdam criteria. It has to be mentioned, the number of the tested patients in all comparative studies was different.

In our study thepatients with HNPCC (N=7), 6 of them (85.7%) had a presence of loss of heterozygosity (LOH) for APC gene, 3 patients (42.8 %) for DCC1 and DCC2, 2 patients (28.4%) for NM23 and 1 patient (14.2%), for p 53 and RB1. These results indicate that there is LOH of tumor suppressor genes for the colorectal cancer located on chromosomes: 5, 13, 17 and 18.

Sasaki et al. (12) researched samples of 22 HNPCC patients. LOH was found on chromosome 5 in 24% cases, on chromosome 14 in 30% cases, on chromosome 17 in 27% cases, on chromosome 18 in 20% and on chromosome 22 in 19% cases. These results indicate that the tumor suppressor genes for colorectal cancer are located on chromosomes 5, 14, 17, 18 and 22.

The analysis of our results show that all 7 patients with HNPCC are highly micro satellite unstable. All patients have microsatellite instability in the area of chromosomes 2p, where the gene hMSH-2 is located, one of mismatch repair genes which are considered as the one with the key role in HNPCC. MSI on BAT 40 was present in 84% in our study and that is characteristic of our sample. So far, DNA variants of BAT 25 have been reported in African Americans with relatively high frequencies and very low in the Japanese population (13).

Ponz et al. (10) tested 18 HNPCC families, and 18 families suspected for HNPCC. Eleven of 18 HNPCC patients showed micro satellite instability (61%) while in the patients suspected for HNPCC only 4 were microsatellite unstable (22%). Three germ line mutations were found in 18 HNPCC families.

Aoltonen et al. (11) prospectively tested colorectal cancer in 509 patients and MSI was present in 63 patterns, what means 12% of patients. The samples of normal tissue in 10 patients had germ line mutation of hMLH 1 and hMSH2 genes.

Henry Lynch et al. (14) came to the conclusion, thatso far there is no clear gene type marker for HNPCC and it is necessary to continue with research. But, beside the existing mutation of mismatch repair genes, which is recessive, there must be one more somatic mutation. That is the reason why we have to do testing on MSI and on tumor suppressor genes.

In our study, from 7 patients with diagnosed HNPCC in the area of the recto sigmoid region, 57.1% of cancers have been located, while in the area of proximal part of colon 42.9% of cancers were located. Analysis of the distribution of cancers, according to Astler-Coller classification, showed that the level C-1 is represented in 42.8% and the level C2 in 71.4% of cases. The presence of metastatic cancer is significant ($p<0,005$). Adenocarcinoma was diagnosed in 71.5% cases, while the Adenocarcinoma mucinosum is presented in 28.5% cases.

Lynch et al. (14) mention that 65-88% of colorectal cancers within the group of HNPCC, occur in the proximal part of the colon. It is significant that there is a greater presence of mucinous cancer within the framework of HNPCC, and it is diagnosed in the lower level of spreading according to Astler – Coller classification. Those patients have a high possibility of developing metachrone cancers, and 18% of such patients at the first diagnoses of cancer have multiple synchronous tumor.

Why is so difficult to identify patients with Lynch syndrome? There are many challenges, mostly technical, that limit the ability to make the diagnosis. The most comprehensive approach would be to perform genetic testing on every patient with colorectal cancer. However such a strategy would be prohibitively expensive and would not identify all cases (15).

This study indicates the importance of usage of Amsterdam's and Bethesda criteria and diagnoses of HNPCC as well as registration of HNPCC families. We understood that if we do not make it now, all colorectal cancers are going to be classified and treated as sporadic. We think that the low rate of incidence of colorectal cancer in our region is not realistic but is a result of the absence of screening programs and a registry of cancer.

It is necessary to form a national registry for colorectal cancer as well as registries for HNPCC families in Bosnia and Herzegovina. The instrument which was used in the study, makes a contribution to their formation. It is necessary to design a screening program for individuals with average and high risk for development of colorectal cancer and colonoscopy should be the screening procedure of choice. It is necessary to do genetic screening of HNPCC families as well as genetic consultation. We have to do

direct sequencing to identify mutations. By diagnosing HNPCC it was possible to do a review of surgical treatment, to test and follow their relatives as the persons with high risk for development of colorectal cancer.

Conclusion

Despite progress in diagnosis, surgery, chemotherapy and radiotherapy, colorectal cancer presents a real challenge to modern medicine due to its high incidence and mortality rates, as well as a number of etiological unknowns.

HNPCC was diagnosed using The Amsterdam and Bethesda criteria in the group of sporadic colorectal cancer in 15% of cases, and exhibited an MSI-H for the chromosome 2p where the hMSH2 mismatch repair gene is localized.

With our research we obtained data which have a local and global significance. The research should be continued and expanded to a larger number of tested persons to make it possible to compare our results with the results of other authors.

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Five years of living donor kidney transplantation at University clinical center Tuzla, Bosnia and Herzegovina, from 1999 through 2004

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Objective To analyze patient and graft survival rate over the first five years of kidney transplantation at University Clinical Center Tuzla, Bosnia and Herzegovina, in the period from 1999 through 2004. **Methods** Transplantations were done after the candidates were evaluated following European Best Practices Guidelines. After open donor nephrectomies Collins solution was used for kidney perfusion. Donor blood vessels were anastomosed end-to-side to recipient external iliac vessels, whereas donor ureter was anastomosed to the recipient bladder with an antireflux technique. Basic immunosupressive protocol was applied in all pateints, with introduction of Basiliximab (Simulect) on the first and fourth postoperative day as the transplantation in our Center progressed. Descriptive statistical analyses along with Kaplan-Meier survival analysis were performed. **Results** The first living donor kidney transplantation started at University Clinical Center Tuzla, Bosnia and Herzegovina on September, 15 1999. There were 52 transplantations performed through 2004. One, two and five-year graft survivals were 96%, 96% and 75%, respectively. One, two and five-year patient survivals were 94%, 94% and 84%, respectively. One, two and five-year survivals of patients with functioning grafts were 92%, 92% and 67%, respectively. **Conclusion** Kidney transplantation survival rates at University Clinical Center Tuzla, Bosnia and Herzegovina from 1999-2004 did not differ from the rates achieved in developed countries worldwide.

Key words: Kidney transplantation, Donor, Rrecipient, Survival rates.

Introduction

There were 1,863 patients on chronic dialysis program and 57 kidney transplants with functioning graft in Bosnia and Herzegovina in 2004 (1). Before the war in Bosnia and Herzegovina 1992-95 kidney transplants were performed at the Transplant institute in Sarajevo. However, its facilities were devastated during the war, but transplantation

has been renewed due to the growing need for that type of renal replacement treatment. The pioneer steps were made after the war in Bosnia and Herzegovina at University Clinical Center Tuzla, where kidney transplants include living related, living unrelated and deceased donor transplants. Living unrelated donor transplantation has not been legally regulated in Bosnia and Herzegovina, despite some sporadic cases and the obvious need for that kind of treatment. The aim of this study was to analyze patient and graft survival rate over the first 5 years of kidney transplantation at University Medical Center Tuzla, Bosnia and Herzegovina, in the period from 1999 through 2004.

Methods

European Best Practice Guidelines for Renal Transplantation and European Society for Organ Transplantation guidelines were followed when preparing the candidates for transplantation (2). Candidates were admitted after having been acquainted with them relevant information on transplantation and collected certain out-patients' medical test resuls. Donor was evaluated first, followed by a recipient. In an operating theatre open donor nephrectomy was done using a flank incision. Collins solution was used for kidney perfusion. Graft biopsy was done and the graft was implanted in contralateral iliac fossa. Donor blood vessels were anastomosed end-to-side to recipient external iliac vessels, whereas donor ureter was anastomosed to recipient blader with an antireflux technique.

Basic immunosupressive protocol (Cyclosporine A, Azathioprin and Prednisolon) was applied in first 28 patients. All other received Cyclosporine A, Mycophenolate mofetil and Prednisolon. Starting with 30st, patient received Basiliximab on the first and fourth post-operative day as the transplantation in our Center progressed. There were no acute rejections in the early postoperative period.

Descriptive statistical analyses were performed. Kaplan-Meier curve was used to perform survival analysis.

Results

First living related donor kidney transplantation at University Clinical Center Tuzla was performed on September, 15. 1999. Totally, 52 transplantations were performed up to 2004 and all were living-related.

Donor characteristics are presented in Table 1.

Three donors had borderline DTPA clearance rates of about 40 ml/min. Nephrectomy was mostly done on the left side, five on the right side, only. There were two renal arteries in five patients. In one case both renal arteries were sewn to the external iliac artery. There were 17 donors older than 60, thereby influencing our results, even though donors met all eligibility criteria (Figure 1).

Recipient characteristics are shown in Table 2.

Recipient age distribution is shown in Figure 2.

There were 8 biopsies of native kidneys performed prior to transplantation. Glomerulonephritis was diagnosed in 28, pyelonephritis in 3, interstitial nephritis in 5, diabetes mellitus in 3, systemic lupus erythematodes in 1, non-differentiated primary kidney disease in 8 and vesicoureteric reflux in 4 cases.

Two recipients underwent nephrectomy on the side of vesicoureteric reflux, whereas one was due to infected reflux. Bladder neck sclerosis was found in one patient and one underwent bladder augmentaton procedure.

Complication in 52 recipients are shown in Table 3.

Table 1 Donor characteristics

Relationship	N	Age (years) X ± SD	*DTPA Clearance rate (ml/min; X± SD)		N of renal arteries >1
			Right kidney	Left kidney	
Mother	24	55,0±11,3	54,8±10,4	53,6±10,5	3
Father	15	61,4±6,9	49,0±6,4	47,8±6,6	1
Sister	5	44,5±6,4	67,0±2,9	65,5±6,4	1
Brother	6	46,0±2,9	54,5±10,6	57,5±12,0	-
Others**	2	38,5±5,5	54,0±4,0	56,5±6,5	-
Total	52	51,1±6,58	55,9±10,1	56,2±8,4	5

*DTPA – diethylen etriamine pentaacetic acid; ** – uncles, aunts and nephews

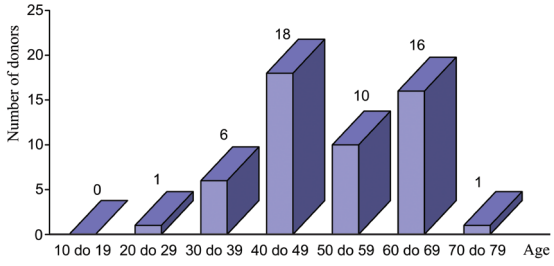

Figure 1 Histogram of donor age distribution

Table 2 Recipient characteristics

Age (years; X ± SD	32,4±8,1
Sex (males/females)	36/16
Time on dialysis (months; X ± SD)	21,5±6,7
Residual kidney function – diuresis	N
0 ml	7
0-100 ml	11
101-500 ml	11
> 500 ml	23
Voiding cystourethrogram	N
Vesicoureteric reflux	4
Small bladder capacity	21
Normal bladder capacity	27

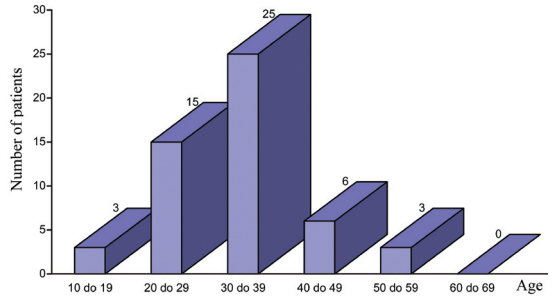

Figure 2 Histogram of recipient age distribution

Table 3 Recipient complications

Complications	Patients N (%)
Acute rejection	10 (19.2)
Venous graft thrombosis	1 (1.9)
Urinom	1(1.9)
Lymphocoella	2 (3.8)
Perirenal hematom	2 (3.8)
Total	16 (30.6)

One graft nephrectomy was done due to venous thrombosis.

Two patients died, one of aneurysm rupture of the thoracic aorta and the other from cardiac arrhythmia.

Average serum creatinine after 5 years of follow-up was 154 ± 22 μmol/l.

Reasons for loss of graft function are shown in Table 4.

Table 4 Reasons for loss of graft function

Reasons for graft loss	Number of patients
Chronic graft nephropathy	4
Primary graft non-function	1
Lung and graft TB*	1
**CMV infection	1
IgA nephropathy	1
Total	8

* Tuberculosis; ** Cytomegalovirus

Figure 3 shows grafts survival at our Center.

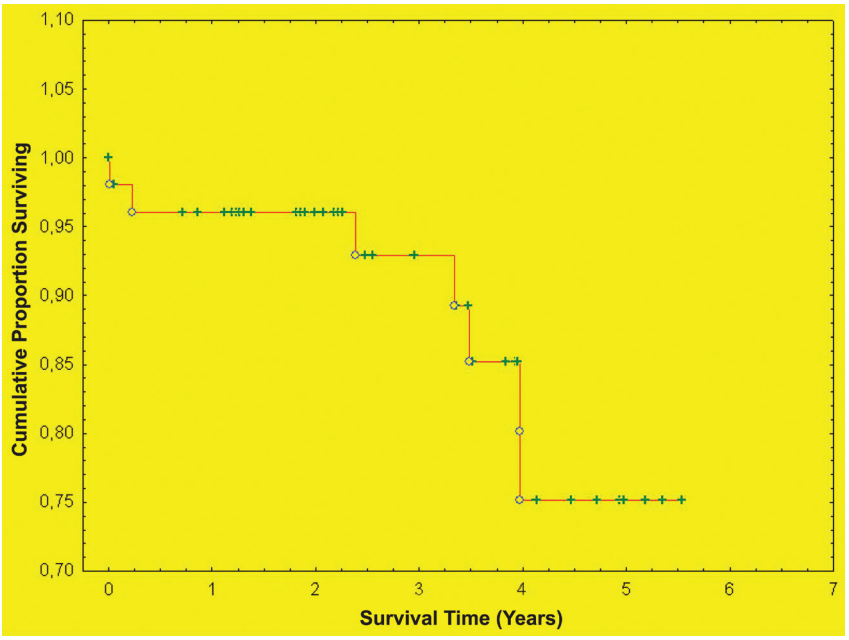

Figure 3. Grafts survival. One, two and five-year graft survivals accounted for 96%, 96% and 75%, respectively

Figure 4 shows patient survival at our Center.

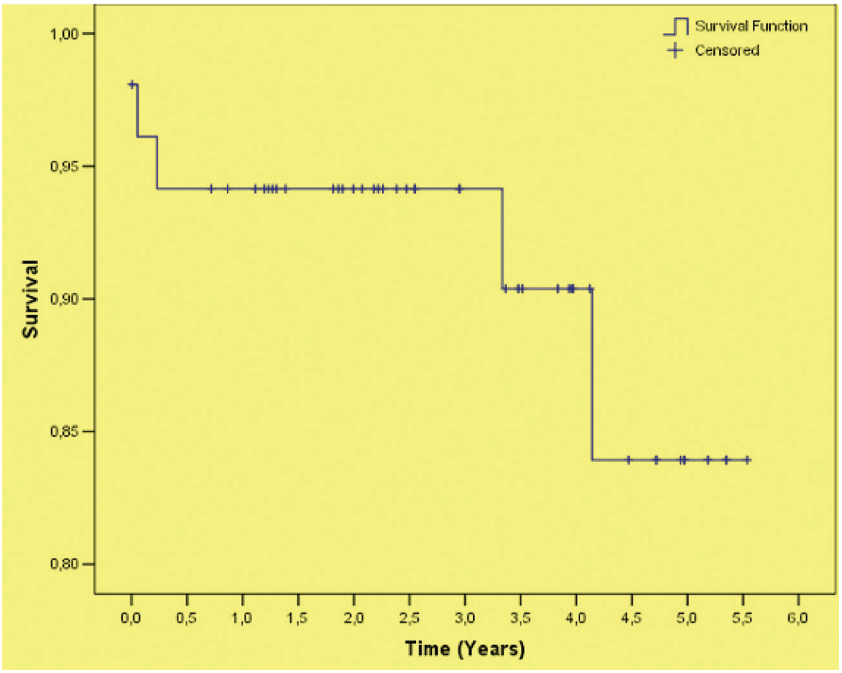

Figure 4. Patient survival. One, two and five-year patient survivals accounted for 94%, 94% and 84%, respectively

Figure 5. shows patients with functioning grafts survival at our Center.

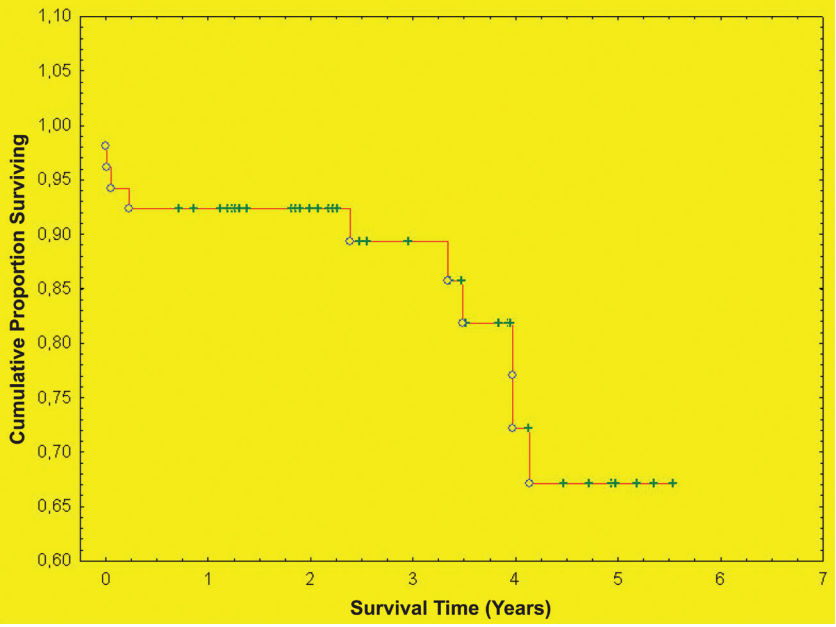

Figure 5 Patients with functioning grafts survival. One, two and five-year survivals of patients with functioning grafts accounted for 92%, 92% and 67%, respectively

There were no complications related to donors over the two years of follow-up.

Discussion

There were 52 living donor kidney transplantations at University Clinical Center Tuzla, Bosnia and Herzegovina, in the period from 1999 through 2004. The one year survival rate at our Center does not differ from the survival rate in Western Europe and USA (3). Five year survival rate of patients with functioning grafts is 67%, which is less than the average of 75%, but any result between 65-75% seems to be acceptable.

One patient underwent pre-transplant nephrectomy, even though reflux was also found in the other kidney, but it was not considered vulnerable to infection.

Bladder augmentation procedure for small bladder capacity was done in one patient. That patient was anuric for 10 years before transplantation.

Since the need for organs is growing, the trend should be toward deceased transplants.

In May, 2005 Parliament of Federation of Bosnia and Herzegovina adopted a law on deceased transplant program applicable to the Federation, only. This program represents the "Spanish model", which has proved most successfull worldwide.

First deceased kidney transplant in Bosnia and Herzegovina was performed at University Clinical Center Tuzla on June, 22 2006. On that occasion the liver was transplanted, too.

Conclusions

Kidney transplantation survival rates at University Clinical Center Tuzla, Bosnia and Herzegovina from 1999-2004 did not differ from the rates achieved in developed countries worldwide. The existing program needs to be updated, particularly with re-

spect to donor selection regarding biological and chronological age.

Aknowledgment

Kidney transplant program at University Clinical Center Tuzla, Bosnia and Herzegovina, was developed in cooperation with General Hospital AKH Wien (Austria). We would like to thank General Hospital AKH Administration for allowing the author of this article to present it at Austrotransplant Congress in Insbruck (Austria) in 2005.

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Telomeres and human disease

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Telomeres are specialized structures at chromosome ends required for chromosome stability maintenance. They consist of a specific repetitive DNA sequence and a set of associated proteins that form a protective structure at chromosome ends. The enzyme telomerase, which is active in stem cells but not in normal somatic cells, synthesizes telomeric DNA sequence. This enzyme is important for cell proliferative potential and most cancer cells have active telomerase. Telomeres are shorter in older individuals than in younger individuals and they may be viewed as a "biological clock". The evidence is accumulating that telomere maintenance plays a significant role in the pathology associated with some human diseases. There are several human genetic diseases that show accelerated shortening of telomeric DNA sequences including Dyskeratosis congenita, Fanconi anemia, ataxia telangiectasia, Nijmegen breakage syndrome, Werner syndrome, Bloom syndrome, pulmonary fibrosis and ataxia telangiectasia like disease. A common feature of these diseases is accelerated telomere shortening due to increased cell turnover that eventually leads to signs of premature ageing. Common diseases lacking an apparent genetic component such as atherosclerosis, heart failure, liver cirrhosis and ulcerative colitis, also show accelerated telomere shortening in affected tissues, that eventually causes tissue specific pathology. Factors that increase cell turnover may be detected by measuring telomere length in the human population and so far several such factors have been identified including: smoking, obesity and exposure to psychological stress. It is likely that future research will reveal an even more extensive role of defective telomere maintenance in human disease and conditions that elevate disease risk.

Key words: Genetic diseases, Telomeres, DNA sequences, Premature ageing.

Introduction

Telomeres are specialized nucleo-protein complexes at the ends of chromosomes identified ~ 70 years ago by Herman Joseph Muller and Barbara McClintock (1, 2). Their pioneering experiments on drosophila and plant cells respectively unravelled the protective properties of natural chromosomal ends, later termed telomeres by Muller using the Greek words telos (end) and meros (part). These simple cytological experiments revealed resistance of telomeres to fusion with either broken chromosomal fragments or other telomeres. The protective property of telomeres is now known as the "capping" function i.e. telomeres provide a protective "cap" for chromosomal DNA against various molecular insults, including nucleolitic degradation, oxidative stress, interaction with other DNA sequences etc.

The molecular dissection of telomeres started in 1970's by identifying the DNA sequence of telomeres in a single cell organism called *Tetrahymena thermphila* (3). This was followed by identification of telomeric DNA sequences in other species including humans and the discovery of telomerase, the enzyme that synthesizes telomeric DNA (4, 5). Subsequent experimental work led to the discovery of an apparent link between the mechanisms that maintain telomeres in our cells and two processes of great importance to human health: ageing and carcinogenesis. It is now clear that telomeric DNA is gradually lost with consecutive cell divisions leading to proposals that telomeres act as a "biological clock" that regulates cell and organismal ageing (6). It is also interesting that telomerase is inactive in human somatic cells but highly active in cancer cells which, owing to telomerase, can proliferate indefinitely, suggesting that telomerase activity is a key requirement for a cell to become cancerous (7).

The above observations were certainly exciting and in the last 20 years numerous studies addressing different aspects of these observations have been published. It has also become apparent that there are several human genetic diseases characterized by premature ageing and predisposition to cancer that show abnormalities in telomere maintenance (see below) thus indicating the medical relevance of telomere biology. This is further substantiated by more recent observations, pointing to a clinically relevant scenario that individuals with susceptibility to cardiovascular diseases may have abnormal telomere metabolism, or that exposure to psychological stress may lead to pathological telomere shortening (see below). Factors such as obesity and smoking are now known to contribute to pathological telomere shortening (see below).

The purpose of this article is to provide a brief overview of the molecular biology of telomere maintenance and address the role of telomeres in human genetic diseases. Furthermore, some common human diseases without an identified genetic component that show defects in telomere maintenance will be discussed. It is also becoming increasingly clear that measurement of telomere length has important prognostic and diagnostic values and some examples will be given to illustrate this point.

Molecular organization of telomeres

As stated earlier, telomeres are specialized structures at chromosomal ends consisting of a specific DNA sequence and a set of associated proteins that bind the sequence. The telomeric DNA sequence in all vertebrates, including humans, is a hexanucleotide, TTAGGG, repeated many times (Fig 1). In human cells the size of telomeric repeat DNA sequences is, on average, between 10 and 15 kb (kilo bases) (4). The size of telomeric DNA sequences varies between individuals and is thought to be genetically regulated. Telomeric DNA is arranged in the form of

a loop known as the T (telomeric) – loop (8) (see also Fig 2). In this configuration the protruding 3' telomeric single strand, the size of which is approximately 100 or more b.p. (base pairs), folds back to form the loop. A set of proteins called sheltretin regulates the arrangement and folding of the T-loop (9). This set consists of six proteins, some of which have affinity for telomeric double stranded DNA sequence (TRF1 and TRF2) and some for telomeric single stranded DNA sequence (Pot1). The purpose of the T-loop is to provide stability for the chromosome. Without this configuration the chromosomal end would be recognized as a DNA double strand break (DSB) by cellular mechanisms that detect and repair DNA damage. The presence of proteins that participate in DSB repair at telomeres, through interaction with shelterin, signifies the importance of interactions between telomeres and DSB repair mechanisms (9). The list of proteins that form shelterin and proteins involved in DSB repair and other types of DNA repair present at telomeres is shown in Table 1.

An important feature of the telomeric DNA sequence is its constant loss with each cell division cycle. It is estimated that this loss is on average ~ 100 b.p. / cell cycle (6). The loss is caused by two mechanisms: the end-replication problem and exonucleolytic degradation. Conventional DNA polymerases that replicate DNA cannot replicate the end of the chromosome properly and this is known as the end-replication problem. The problem was first identified by Olovnikov (10). He proposed that the loss of telomeric DNA may act as a "biological clock", a counting mechanism which may signal cells how many times to divide. When the loss eventually causes a near complete exhaustion of telomeric DNA, this will signal the cell cycle stop. The affected cell will no longer be able to divide and it will be replaced by a younger cell with longer telomeres. Experiments have confirmed the view of telomeres

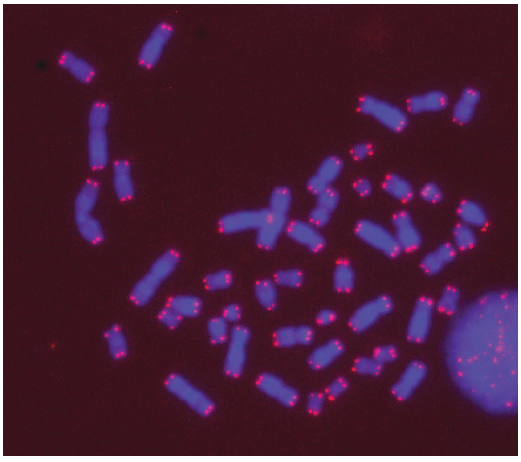

Figure 1 A human cell showing 46 chromosomes (blue) after fluorescence in situ hybridization with the telomeric TTAGGG probe (red).

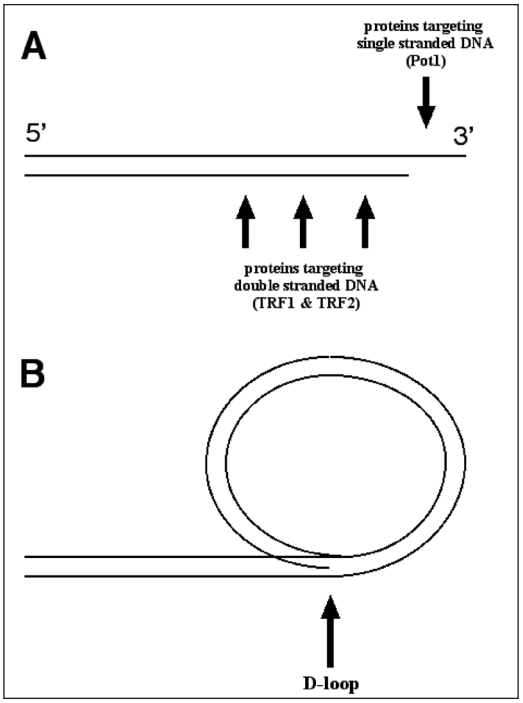

Figure 2. Molecular structure of telomeres. A. Linear telomeric DNA with a 3' single stranded overhang, the size of which is ~ 100 base pairs. B. This single strand overhang folds back, invades the DNA double strand to form a local DNA triplex (three strands) known as D-loop (Displacement loop). The whole loop structure is called T-loop (Telomeric loop). The protein named TRF2 enables formation of a local DNA triplex.

Table 1 Proteins that associate with telomeres. For disease abbreviations see text

Protein	Role	Associates with	Human disease
TRF1	Shelterin	Double stranded TTAGGG sequence	Not known
TRF2	Shelterin	Double stranded TTAGGG sequence	Not known
Pot1	Shelterin	Single stranded TTAGGG sequence	Not known
Rap1	Shelterin	TRF1/2	Not known
TPP1	Shelterin	TRF1/2	Not known
Tin2	Shelterin	TRF1/2	Not known
Tank 1	Shelterin associate	TRF1/2	Not known
Tank 2	Shelterin associate	TRF1/2	Not known
Parp 2	DNA repair	TRF2	Not known
Ku	DNA repair	TRF1/2	Not known
DNA-PKcs	DNA repair	TRF1/2	Not known
Rad51D	DNA repair	TRF1/2	Not known
WRN	DNA helicase	TRF1/2	Werner syndrome
BLM	DNA helicase	TRF1/2	Bloom syndrome
MRE11	DNA repair	TRF1/2	Ataxia telangiectasia like disease
NBS1	DNA repair	TRF1/2	NBS
ATM	DNA damage response	TRF2	AT
TERC/TERT/Dyskerin	Telomerase	T-loop	DC, AA

as the "biological clock". For example, telomeres in older individuals are shorter than telomeres in younger individuals (6). However, the end-replication problem is not the only source of telomeric DNA loss. The enzymes with exonucleolytic activity must prepare the T-loop substrate (protruding single stranded telomeric sequence) after each DNA replication cycle and the consequence of the activity of these enzymes is the loss of telomeric DNA (11). Therefore, the end replication problem and exonucleolytic activity will eventually cause degradation of telomeric DNA, after multiple cell divisions, to the extent that the affected cell will no longer be able to divide.

Telomere shortening is an important tumour suppressor mechanism which strictly regulates tissue homeostasis. Cells forming tumours differ from normal somatic cells (with the exception of stem cells) in their growth properties. Normal somatic cells can divide up to a maximum of ~ 50 times, after which they exit the cell cycle. In contrast, tumour cells can divide indefinitely. What is the mechanism that confers this indefinite cell division potential of tumour cells? After many years of research, scientists discovered that tumour cells acquire the capacity to prevent telomere loss and subsequent telomere shortening. This capacity is provided by a specialized enzyme termed telomerase, which synthesizes telomeric DNA and by doing so stops telomeric DNA loss (6). Telomerase is a complex enzyme consisting of its own RNA component known as TERC, the catalytic protein component known as TERT and a protein called Dyskeryn. Experiments have shown that up to 85% tumours have active telomerase, whereas normal somatic cells lack telomerase activity (6). (The only normal somatic cells that have relatively high levels of telomerase are stem cells. This is understandable given that stem cells must have high growth potential in order to maintain tissue homeostasis.) Those remaining 15% of tumours that lack telomerase use another mechanism to stop telomere loss, known as ALT (Alternative Lengthening of Telomers) (6). Inhibiting telomerase or ALT mecha-

nisms in cancer cells constitutes a promising avenue for cancer therapy.

The medical relevance of telomere biology

As indicated above, there are two clear areas of telomere biology relevant to medicine. These include mechanisms of human ageing and mechanisms of carcinogenesis. The area of telomere biology related directly to cancer is beyond the scope of this review.

Although the current understanding of mechanisms that regulate human ageing is limited, it is clear that telomere biology represents a significant contributing mechanism. This is recognized by the fact that one of the theories proposed to explain mechanisms of ageing, known as "Telomere theory of aging", directly implicates telomere shortening as a cause of ageing or senescence in human cells (6). It is not yet completely clear whether the accelerated process of cell senescence will cause accelerated ageing of an organism but the evidence is accumulating that this may be true.

According to the "Telomere theory of ageing", most somatic cells in the human body must have a limited capacity to divide, known as the Hayflick limit (6). In 1960s Leonard Hayflick concluded, after his experiments with human somatic cells, that they cannot divide more than ~ 50 times *in vitro*. He argued that this mechanism regulates tissue homeostasis, but he did not have an explanation as to how this mechanism may work. The explanation was provided after the discovery of telomerase and improved understanding of telomere maintenance mechanisms. As discussed above, telomeric DNA is progressively lost after each cell division cycle, until the loss signals the end of cell division potential. Experiments have shown a good correlation between the Hayflick limit and telomere shortening, thus implicating the latter as a key regulator of the "biological clock". The key evidence supporting the essential role of telomeres in the "biological clock" came from experiments in which forced activation of telomerase in otherwise normal human somatic cells resulted in the acquired capacity of these cells to divide indefinitely (6). This means that when physiological telomere shortening is stopped, human cells in effect become "immortalized" and the key tumour suppressing mechanism that regulates tissue homeostasis is lost. Further evidence in support of the "Telomere theory of ageing" was provided by observations that human diseases characterized by premature ageing symptoms show abnormal telomere metabolism (see below). Some of these diseases will be described in more detailed below and evidence that links premature ageing symptoms and defective telomere maintenance will be highlighted.

Human genetic diseases with dysfunctional telomere maintenance

The common features of diseases described in this section include: a) symptoms associated with premature ageing and b) some form of abnormality in telomere maintenance that may manifest either as accelerated telomere shortening, or loss of telomere capping function. Research has shown that defects in telomere maintenance associated with these diseases contribute significantly to disease pathology (see below).

Dyskeratosis congenita (DC)

DC is an inherited bone marrow (BM) failure syndrome with clinical features becoming apparent in childhood (12). BM failure can be defined as the inability of bone marrow to provide a sufficient number of circulating blood cells. This suggests that hematopoietic stem cells that reside in the BM, function abnormally in DC. Other symptoms of DC, including abnormal skin pigmentation, nail dystrophy

and leucoplakia are consistent with stem cell failure in organs such as the skin.

Molecular and cell biology research has provided a reasonable explanation as to how failure in HSCs leads to symptoms of DC. It is now clear that DC is caused by mutations in genes encoding components of telomerase and this ultimately leads to abnormally short telomeres in DC patients (12). Abnormally short telomeres are ultimately responsible for diminished growth potential of the stem cells that maintain highly proliferative tissues, such as BM and skin. This also strongly indicates that telomerase activity is an essential requirement for normal proliferation of stem cells.

Genetic research has identified three forms of DC (12). The first known form of DC was the so called X-linked dominant DC because the gene responsible was located on the X chromosome. The gene called *DKC1* was eventually identified as responsible for this form of DC. It encodes a protein called dyskerin. Initially, it was thought that this protein is the key to the biogenesis of ribosomes, until the link with telomerase was discovered. Dyskerin is now known to be a component of telomerase. Given that some DC patients did not show mutations in *DKC1* it became obvious that other genes may be involved in this disease. Through the so called linkage analysis, a family with many members affected by DC was identified. Genetic research revealed that a gene in the region of chromosome 3q, the same region where the *TERC* gene resides, was responsible for this form of DC. Further investigation revealed *TERC* mutations in the family, thus leading to the conclusion that a new form of DC, now known as autosomal dominant DC, is due to mutations in *TERC*. Subsequent studies revealed that abnormally short telomeres are one of the key molecular features of DC, suggesting that this disease is a disease of abnormal telomere maintenance (12). During the course of these studies another form of DC was discovered, now known as autosomal recessive DC. However, the gene or genes responsible for autosomal recessive DC have not been identified yet. It is likely that these genes will be involved in telomere maintenance. Finally, a group of DC patients, with mutations in the *TERT* component of telomerase, has recently been described, adding further support to the view of abnormal telomere maintenance as the key molecular cause of DC (12). Patients with *TERT* mutations are classified as the autosomal dominant variant of DC.

Fanconi anemia (FA)

Similarly to DC, FA is an inherited BM failure syndrome (13). Patients are typically diagnosed in the first decade of life and many will die as young adults as a result of BM complications. In addition, FA patients show extreme predisposition to cancer and many will die as a result. The disease is inherited mainly in an autosomal recessive fashion. Apart from BM failure, many patients show somatic abnormalities, including skin, skeletal, neurological, cardiovascular and other abnormalities.

Cells from FA patients show high sensitivity to the chemicals that cause DNA cross-links, such as mitomycin C (MMC) and diepoxybutane (DEB), which manifest by the presence of high level of chromosomal abnormalities in the patients' cells relative to control cells. This is the key diagnostic test for FA. Genetically, FA is a very complex disease, with at least 12 different genes involved, known as FANCA to FANCM (13). All genes are involved in DNA damage response, in particular the pathway that regulates response to DNA crosslinking agents.

FA patients have significantly shorter telomeres than corresponding control patients (14, 15). In addition, FA cells show increased incidence of end-to-end chromosome fusions as a result of accelerated

telomere shortening. It can be argued, as in the case of DC, that telomere shortening observed in FA patients causes the inability of HSCs to replenish BM, thus leading to symptoms of BM failure.

Ataxia telangiectasia (AT) and Nijmegen breakage syndrome (NBS)

AT and NBS are genetic diseases characterized by increased sensitivity of patients to ionizing radiation, high incidence of cancer and chromosomal instability (16). In contrast to DC and FA, only one gene is responsible for AT and NBS respectively. The genes are known as ATM (AT mutated) and *NBS1*. Both genes are involved in DNA damage response mechanisms. ATM is a key signalling molecule, activated by the presence of DNA damage in affected cells, whereas NBS1 is part of a protein complex called MRN consisting of NBS1 and two more proteins, MRE11 and Rad50 (16). Both diseases are inherited in an autosomal recessive fashion.

There are clear abnormalities in telomere maintenance in cells from AT and NBS patients (16). Telomere shortening is evident in both cases. However, in the case of AT cells, telomere shortening is accompanied by increased incidence of end-to-end chromosome fusions and the presence of extrachromosomal telomeric fragments, whereas these are absent in NBS cells. Both proteins, ATM and NBS1, physically interact with the components of shelterin, thus indicating close cooperation between DNA damage response mechanisms and telomere maintenance. Genetically deficient mice, defective in ATM, also show alterations in telomere maintenance. It is important to stress that all diseases mentioned so far, DC, FA, AT and NBS are considered premature ageing syndromes as patients show many features of premature ageing. This provides a formal link between accelerated telomere shortening and ageing at the clinical level.

Werner syndrome (WS) and Bloom syndrome (BS)

WS is a premature ageing syndrome characterized by signs of ageing at an early age, usually in the second decade of life, that include grey hair, alopecia, skin ageing, cataracts, ischemic heart disease etc. (17). Affected individuals also show high levels of inflammatory diseases such as atherosclerosis and type 2 diabetes, as well as a high incidence of cancer. The gene responsible for WS is called *WRN*. It encodes a protein with helicase domain involved in DNA metabolism including replication, recombination and repair. Fibroblasts isolated from WS individuals show premature senescence in vitro that can be rescued by ectopic expression of telomerase (17). This implicates dysfunctional telomere maintenance as a cause of WS associated pathology. Consistent with this possibility, biochemical experiments revealed direct interaction between WRN and telomeric proteins such as TRF1 and TRF2. It is therefore likely that abnormal WRN will affect the so called telomere capping function and lead to increased telomeric fusions and subsequent chromosomal instability.

BS shows some similarity to WS. The protein affected in BS, known as BLM, is a helicase like WRN and BS patients show limited signs of premature ageing, such as early menopause and elevated rates of cancer. It is interesting that BLM also interacts with telomeric proteins, such as TRF1 and TRF2 (18). Published studies that directly examined the state of telomere maintenance in BS patients are not yet available. However, mice defective in BLM and TERC show pathology associated with telomere loss that is directly attributed to dysfunctional BLM (18). It is therefore possible that BS patients may show some form of telomere dysfunction. Another interesting feature of BLM is its involvement in the ALT pathway of telomere maintenance (19).

AT like disorder (ATLD), pulmonary fibrosis (PF) and aplastic anemia (AA)

The evidence is accumulating that telomere defects may exist in the above three disease. A subset of AA patients shows mutations in *TERT* and *TERC* genes (20). These mutations cause accelerated telomere shortening and could cause the premature death of AA patients. Mutations in *TERT* and *TERC* genes have been identified in patients with PF (20). PF is characterized by lung scarring, which eventually leads to respiratory failure and the disease could be lethal. ATLD patients show mutations in the gene encoding the Mre11 protein. The protein is part of the MRN complex (see above) known to be involved in DNA damage response. One of the features of ATLD is accelerated telomere shortening (20). All diseases described in this section are summarized in Table 2.

Common human diseases with defective telomere maintenance

Research in the last decade revealed several common human diseases that show alterations in telomere maintenance. Common diseases are defined as diseases occurring in the human population with a relatively high frequency, but lacking an apparent inheritance pattern. This does not mean that common diseases completely lack the genetic component. It is likely that common diseases are caused by multiple genes, the individual effect of which is small, and as a result the inheritance pattern is not obvious, as in the case of classical genetic diseases.

One of the first common diseases in which alterations in telomere maintenance were observed was atherosclerosis. Atherosclerosis is now believed to be the result of systemic chronic inflammation. Given that chronic inflammation requires increased cell turnover, it has been argued that patients with atherosclerosis may have abnormally short telomeres in some or all tissues. Consistent with this possibility it was shown that atherosclerosis patients have significantly shorter telomeres in their leukocytes relative to control individuals (21).

Similarly, investigation of heart muscle cells, known as myocytes, from patients with heart failure, revealed a 25% reduction in telomere length relative to their control counterparts (22). Furthermore, myocytes in affected patients showed a higher level of apoptosis, programmed cell death, as well as alterations in expression of the telomeric protein TRF2 relative to cells from control individuals (22). Given that heart failure is caused by myocyte deficiency, which even-

Table 2 Genetic diseases with dysfunctional telomere maintenance

Disease name	Disease main characteristics	Protein affected	Effect on telomere maintenance
Dyskeratosis congenita	Bone marrow failure	TERC, TERT, Dyskerin	Accelerated telomere shortening
Fanconi anemia	Bone marrow failure	At least 12 FANC proteins	Accelerated telomere shortening, chromosome fusions
Ataxia telangiectasia	Radiation sensitivity syndrome	ATM	Accelerated telomere shortening, chromosome fusions
Nijmegen breakage syndrome	Radiation sensitivity syndrome	NBS1	Accelerated telomere shortening
Werner syndrome	Premature ageing syndrome	WRN	Accelerated telomere shortening
Bloom syndrome	Defective DNA damage response	BLM	Effect on ALT
Ataxia telangiectasia like disease	Defective DNA damage response	MRE11	Accelerated telomere shortening
Aplastic anaemia	Anaemia	TERC, TERT	Accelerated telomere shortening
Pulmonary fibrosis	Respiratory disease	TERC, TERT	Accelerated telomere shortening

tually leads to failure in the heart pumping function, the results of the above study established a formal link between telomere maintenance and heart diseases. Furthermore, more recent studies indicate that telomere length is a good predictor of the risk for cardiovascular diseases. The highest risk of cardiovascular diseases is associated with the shortest telomeres in affected patients (23).

Another common disease, in which telomeres are affected, is liver cirrhosis. Irrespective whether cirrhosis is caused by viral hepatitis, increased consumption of alcohol, autoimmunity or cholestais, telomeres were always significantly shorter in hepatocytes from affected patients than in hepatocytes from control individuals (24). In contrast, telomere length in leukocytes from cirrhosis patients was normal (24). The study therefore supports the view that chronic hepatocyte damage and induced hepatocyte regeneration accelerate telomere shortening in hepatocytes. The study also implies that restoration of telomere length in hepatocytes from patients affected by cirrhosis could constitute a potential therapy for this disease.

Ulcerative colitis is the last example of a common human disease in which telomere maintenance is affected (25). This is a chronic inflammatory disease of the colon, associated with a high risk of colon cancer. Accelerated telomere shortening and associated chromosomal abnormalities, such as end-to-end chromosome fusions, are apparent in non-dysplastic mucosal cells taken from individuals affected by the disease (25).

In summary, all the above common diseases are associated with accelerated telomere loss. It is likely that the accelerated telomere loss is caused by increased cell turnover which eventually reduces the regenerative capacity of affected tissues, most likely through the failure of stem cells to maintain tissue homeostasis (see Fig. ?).

Telomere length as a prognostic marker

In view of the fact that human diseases, characterized by an increased cell turnover in highly proliferative tissues, show accelerated telomere shortening (see above) telomere length can serve as a marker capable of detecting any factor that stimulates cell turnover. It is likely that such factors will cause damage to cells and as a result stimulate tissue processes which eventually replace damaged cells by healthy cells through increased proliferative activity of stem cells. Although stem cells have a higher proliferative potential than differentiated cells, this potential is not unlimited and increased cell turnover constitutes a risk factor that accelerates ageing and may cause early mortality. In line with this prediction, telomere length was reported to be a good predictor of mortality rate among people aged 60 or more (26). This study has important implications, as the factors that lead to increased cell turnover, and thus accelerated telomere shortening, can be identified. Once such factors are identified, lifestyles can be changed accordingly, to avoid exposure to identified risk factors.

Factors that cause accelerated telomere shortening include obesity, smoking and exposure to psychological stress. Analysis of telomere length in leukocytes from a large group of women revealed an inverse association between the number of cigarettes smoked and telomere length (telomeres were shorter in smokers) (27). A similar inverse correlation was observed between the body mass index (BMI) and telomere length i.e. the higher the BMI, the shorter the telomeres (27). In a separate study, telomere length was measured in a group of care-giving mothers whose children were chronically ill. The chronicity of care-giving was considered to be a significant stress factor. There was an inverse correlation between chronic-

ity of care-giving and telomere length i.e. the higher the number of care-giving years, the shorter the telomeres (28). In addition, telomerase levels were lower in women exposed to stress longer.

It is interesting to note that there are numerous causes of psychological stress. The question is whether each one of these causes is capable of causing accelerated telomere loss. The answer to this question may be positive according to a recent study, which revealed shorter telomeres in people with lower socio-economic status than in their counterparts with higher socio-economic status (29). The argument here is that low socio-economic status is associated with a high level of psychological stress. However, this study received some criticism and its conclusions were questioned (30, 31). In addition, another study was designed to detect whether telomeres and telomerase are affected by psychological stress arousal and risk of cardiovascular disease. The study revealed that low telomerase activity in leukocytes was associated with major risk factors for cardiovascular diseases, including poor lipid profile, hypertension and abdominal adiposity (32).

Conclusion

Only 20 years ago the structure and function of telomeres were relatively poorly understood and there was no indication that telomere metabolism may play a significant role in understanding the mechanisms of human diseases. As explained above there are now a growing number of human genetic and common diseases that show alterations in telomere maintenance and these alterations contribute significantly to disease pathology. A common feature of diseases with affected telomere maintenance is premature ageing. Signs of premature ageing in these diseases can be explained by increased cell turnover, which leads to the increased proliferative capacity of stem cells and accelerated telomere shortening in differentiated somatic cells. Factors that increase cell turnover can be detected by measuring telomere length in the human population and so far several such factors have been identified, including smoking, obesity and exposure to psychological stress. Taken together, these observations suggest that telomere maintenance plays a significant mechanistic role in human disease pathology.

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Meningoencephalitis in splenectomized patient caused by concurrent *Streptococcus pneumoniae* and Herpes simplex virus infection

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In this paper we have described a meningoencephalitis in splenectomized patient caused contemporaneously by *Streptococcus pneumoniae* and Herpes simplex virus. The unusual course of pneumococcal meningitis accompanied with worsening of the patient's condition and repeated comatous status directed clinical diagnosis to the new etiologic agent of meningoencephalitis. After antibiotic and antiviral therapy the patient fully recovered.

Key words: Concurrent, Meningoencephalitis, Herpes, Pneumococcal.

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Introduction

Simultaneous infections of the central nervous system caused by different pathogens are very rarely seen even in immunocompromised patients. In the adult population up to 60 years of age, *Streptococcus pneumoniae* is responsible for about 60% of cases of acute bacterial meningitis (1). Splenectomy is a predisposing factor for bacterial infections especially caused by *Streptococcus pneumoniae* (2, 3) Herpes simplex encephalitis is acute necrotising encephalitis with the highest mortality rate and is the most common encephalitis occuring in immunocompetent patients (4, 5). In this paper we have described meningoencephalitis in a splenectomized patient caused contemporaneously by *Streptococcus pneumoniae* and Herpes simplex virus. There is no similar report in the current medical literature.

Case report

M.M. 41-year old man was referred to our hospital after 24 hours of fever, sudden frontal headache, nausea, vomiting and progressive mental confusion. On admission, he was unconscious, with high fever and nuchal rigidity. There were no neurologic signs of lateralisation but skin abdominal reflex function was absent. Lung and heart findings were normal, TA 100/70 mmHg, puls 96/min. He had a history of splenectomy sixteen years ago, and he was not received pneumococcal vaccine. First lumbar puncture performed on the day of admission showed purulent cerebrospinal fluid (CSF) with 151 cells/mm^3, predomination of neutrophiles, undetectable level of glucose and high protein level of 2,2 g/l. Blood and CSF cultures have shown presence of *Streptococcus pneumoniae*. Cultivation of microorganisms was performed in Bacteriology Department of Military Medical Academy. Blood culture was performed on blood agar and CSF culture on chocolate agar. Patient treatment was started immediately with ceftriaxone 4g/day intravenously along with the antiedematous therapy. On the second day of the therapy, the patient become afebrile, and he was in good condition,conscious, but vesicles appeared on patient lips. On the sixth day, the patient becamefebrile again, he had difficulties with speech and his state of consciousness was worsened resulting in coma again. Repeated lumbar puncture has showed blood in CSF with 1827 cells/mm^3 (predominantly lymphocytes), decreased glucose level of 1.9 mm/L (glucose in blood 5.7 mmol/L) and 1.6g/L of proteins. Cultivation CSF for presence of Herpes simplex group viruses was performed in Virology Department of Military Medical Academy and were positive after six days in both CSF samples.

Acyclovir (1,5 g daily) was introduced into the therapy simultaneously with continuous administration ceftriaxon and antiedematous drugs. Two days after introduction of antiviral therapy the patient became conscious, his neurologic findings revealed-motor aphasia, ptosis of the right eyelid, hemiparesis of the right facial nerve and pyramid deficit of the right side of the body.

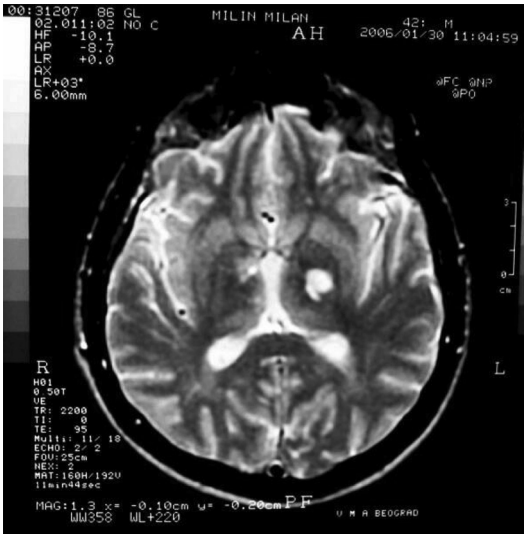

Figure 1 Typical focal lesions in basal ganglia

Magnetic resonance imaging of brain has showed typical focal lesions in basal ganglia (Figure 1).

After two week successive medication and the physical rehabilitation, the patient was able to walk by himself and speak much better.

Discussion

Different types of immunodeficiencies are predictive for infections even of central nervous system. Defects in humoral immunity could lead to recurrent infections with encapsulated bacteria such as *Haemophilus influenzae* or *Streptococcus pneumoniae* (6). Immunodeficiency of T cells is found most commonly accompanying HIV infection, neoplastic disease, corticosteroids and cytotoxic agents use and is mainly associated with infection with intracellular pathogens (*M. Tuberculosis,* Herpes zoster virus, Herpes simplex virus, Cytomegalovirus *Cryp-*

tococcus etc.) (6). There was no evidence of congenital and acquired immunodeficiency in our patient except splenectomy.

Splenectomized patients are likely to develop addiction to septic conditions caused by incapsulated microorganisms, in the first place by *S. pneumoniae*. According to some reports splenectomized patients have twelve times higher risk for developing severe sepsis comparing to patients with spleen (7). A sepsis caused by *S. pneumoniae* in splenectomized patients occurs abruptly with possible development of acute meningitis (8). Due to the rapid development of meningitis, the cerebrospinal fluid could be clear, without cells or with a small number of cells, causing diagnostic confusion.

The viral genome of Herpes simplex virus usually persists as a latent infection in the trigeminal ganglion, and reactivation could be caused by a number of stimuli including febrile illnesses, menstruation, sunlight, stress and immunosuppression (9). There is experimental evidence of latent herpes simplex reactivation after pneumococcal pnemonia in mice (10). The virus has a predilection for temporal lobes of the brain, but extra temporal involvement could be found in as many as 55% of patients (11). Focal changes are identified especially in older patients (12). Concurrent infections of the central nervous system with microorganisms of quite different taxa are extremely rare. Infections have been described with two bacterial species(13), but CNS infections caused by concurrent bacterial and viral microorganisms have not been reported so far. Unusual course of pneumocoocal meningitis accompanied with worsening of the patient's condition and repeated episodes of coma directed clinical diagnosis to the new etiologic agent of meningoencephalitis. In conclusion, the unusual clinical course of bacterial meningitis sometimes could indicate a concurrent viral infection.

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Familial breast cancer: recent advances

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The majority of breast cancer cases are so called sporadic cancers, which do not have a strong genetic component. However, approximately 27% of breast cancer cases are inherited or familial cancers that result from inheriting pathogenic mutations in specific genes. Two of these genes, namely *BRCA1* (BReast CAncer 1) and *BRCA2*, confer a high risk for breast cancer and are known as high penetrance genes. At least 12 more genes associated with breast cancer risk have been identified so far. However, these genes are low penetrance genes that carry a much lower risk for breast cancer than *BRCA1* and *BRCA2*. Four of these low penetrance genes have been identified recently by a large research consortium led by a research group from Cambridge University. It is likely that future research will uncover additional low penetrance breast cancer predisposing genes.

Introduction

The first indication that cancer may be a genetic disease was provided by the influential German scientist, Theodor Boveri, in his celebrated work "Zur Frage der Entstehung Maligner Tumoren" ("The Origin of Malignant Tumours") published in 1914 (1). Although the concept of a gene was unknown at that time, Boveri correctly assumed that chromosomes were carriers of genetic information. His insightful observations of chromosomal abnormalities in tumor cells enabled him to propose that predisposition to cancer may be inherited by inheriting a copy of a chromosome (gene) that is not able to suppress tumor growth efficiently (1).

More recent studies have largely confirmed Boveri's prediction of genetic predisposition to cancer (2). For example, in 1971 Knudson proposed a two-step mutation model of cancer (3). The model is based on

the assumptions that (a) most cancers develop from a single cell and (b) at least two mutational events are required. The model predicts that all cancers can be classified into two categories: 1. sporadic cancers arising as a result of chance (caused by a combination of genetic and environmental factors) and 2. inherited or familial cancers (caused by genetic factors). Both categories involve the same genetic changes or mutations, but the difference between them is in the timing of the mutation's occurrence. In the case of familial cancers individuals are born with the first mutation and all cells in their body carry this mutation. The second mutation is acquired after birth in a relevant somatic cell of these individuals. By contrast, in the case of sporadic cancers both mutations are acquired after birth in somatic cells. Therefore, the chances of two mutations occurring independently in the same somatic cell during the lifetime of an individual (sporadic cancer) are significantly lower than a single mutation occurring in a somatic cell in which another mutation already exists (familial cancer). According to this model, familial cancers should affect younger individuals and can be found at multiple sites in their bodies, whereas sporadic cancers should be confined to older individuals and should appear only at a single site in their bodies. Numerous studies have shown that the Knudson model, which stems from Boveri's early predictions, is applicable to childhood cancers, but also to adult cancers, including breast cancer.

The focus of this article is the familial form of breast cancer. In order to fully appreciate the differences between sporadic and familial forms of breast cancer, basic epidemiological facts will be briefly presented, followed by a more focused description of the molecular mechanisms underlying the familial form of breast cancer. Significant advances in the current understanding of these mechanisms have recently been made (4) and these advances will be briefly described.

Basic facts about breast cancer

According to US estimates, approximately 12% of women will suffer from breast cancer (Fig. 1). Most breast cancers are sporadic and occur with a frequency of approximately 73% (Fig. 1). Sporadic breast cancers are characterized by the lack of family history of disease i.e. no two first-degree relatives in a family are affected. Sporadic breast cancers rarely affect younger women and the cases of the bilateral form of disease (affects both breasts) are rare.

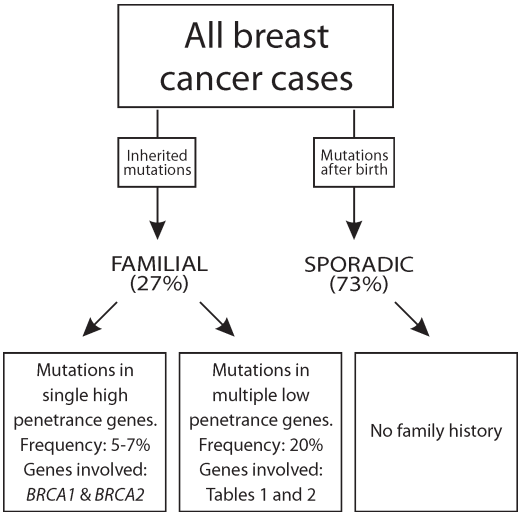

Figure 1. Frequencies of sporadic and familial cancers according to US estimates (for more details see: http://envirocancer.cornell.edu/FactSheet/General/fs48.inheritance.cfm)

The remaining 27% cases of breast cancer are familial cancers (Fig. 1). This type of breast cancer is characterized by a clear family history of disease, which increases a woman's chance of having the disease up to 2-3 times. In addition, patients with familial breast cancer are significantly younger at the time of diagnosis and have a higher frequency of bilateral disease in comparison with their sporadic breast cancer counterparts.

Apart from these epidemiological differences between the two forms of breast

cancer, there are no other obvious differences that affect the clinical and pathological presentation of the disease (5). This is in line with the Knudson's two-step mutation model predicting the same genetic changes or mutations in sporadic and familial cancers. Given that the same genetic events occur in both types of cancer, the underlying molecular mechanism should be the same, thus leading to similar clinical and pathological presentations.

How was the familial form of cancer identified? Researchers realized very early that some breast cancers tend to cluster in families, i.e. multiple first-degree relatives (mother-daughter; sisters) were affected. This suggested a heritable component of the disease. The usual research strategy in such cases is to construct a family tree or a pedigree, collect blood samples from as many family members as possible and subject these samples to genetic investigation. In the early 1990's, genetic analyses of multiple breast cancer families revealed a common gene located on chromosome 17q that was affected (mutated) in some but not all families. This gene was named *BRCA1* (*BReast CAncer gene 1*). After intensive competition between several American and European laboratories, the gene was identified in 1994 (6). Another breast cancer predisposing gene was identified soon after that and named *BRCA2* (7). *BRCA1/2* genes are affected (mutated) in only ~25% of familial breast cancer cases, suggesting involvement of other genes in this form of disease.

BRCA1/2 genes are called "highly penetrant" genes. In medical genetics penetrance is defined as the ability of a gene to express itself, resulting in specific symptoms. Highly penetrant genes will always cause symptoms, irrespective of the environmental effects. By contrast, low penetrance genes produce symptoms only sometimes and can be affected by environmental factors. Therefore, women born with *BRCA1/2* mutations have a high chance of suffering from breast cancer during their lifetime.

Numerous studies have failed to identify another highly penetrant *BRCA* gene, but have instead identified several low penetrance genes involved in familial breast cancer. The full list of low penetrance genes identified by early 2007 (reviewed in reference 8) is shown in Table 1. There are some epidemiological differences between high and low penetrance genes. High penetrance genes, such as *BRCA1/2,* affect many family members and greatly increase breast cancer risk. By contrast, low penetrance genes do not affect as many family members as high penetrance genes and they confer lower cancer risk than high penetrance genes. *BRCA1/2* and the low penetrance genes shown in Table 1 cause approximately 50% of familial breast cancer cases, suggesting that additional low penetrance genes must be involved in this form of disease.

A new study, published in the prestigious journal "Nature" (4), identified several new low penetrance genes using an approach based on recent advances in dissecting the human genome. This approach and the genes identified will be described below. Before that, some basic facts about *BRCA1/2* and low penetrance genes shown in Table 1 will be briefly presented.

Functions of genes involved in familial breast cancer

BRCA1/2 are genes involved in DNA damage response. DNA damage response is the network of cellular mechanisms activated when cells or organisms are exposed to genotoxic stress. These mechanisms typically recognize DNA damage, signal the presence of DNA damage to other relevant molecules, block the cell cycle to allow cells to repair the damage and finally repair the damage via numerous DNA repair pathways. *BRCA1/2* genes are involved in the repair of DNA

Table 1 Familial breast cancer genes as of January/February 2007 according to reference number 8

Gene	Penetrance	Function	Association with other diseases
BRCA1	High	DNA damage response	-
BRCA2	High	DNA damage response	Fanconi anemia
PALB2	Low	DNA damage response	Fanconi anemia
BRIP1	Low	DNA damage response	Fanconi anemia
ATM	Low	DNA damage response	Ataxia telangiectasia
NBS1	Low	DNA damage response	Nijmegen breakage syndrome
RAD50	Low	DNA damage response	-
CHEK2	Low	DNA damage response	-
P53	Low	Tumor suppressor	Li-Fraumeni syndrome
PTEN	Low	Tumor suppressor	Thyroid and colon cancer

double strand breaks through interactions with DNA repair proteins such as Rad51, or interactions with DNA damage signalling molecules such as ATM. The importance of *BRCA1/2* genes in DNA damage response is reflected in the fact that cells defective in these genes show high levels of spontaneous chromosomal abnormalities (9).

It is interesting that *BRCA2* has recently been linked to a human disease – Fanconi anemia (FA). FA is a complex genetic disease, with at least 12 genes known to be involved. The key molecular feature of FA is inability of cells from patients to cope with genotoxic stress, especially the stress caused by the so-called DNA cross-linking agents, such as MMC (mitomycin C) and DEB (diepoxybutane). Sensitivity of FA cells to MMC and DEB suggests inherited failure in DNA damage response in FA patients. All known FA genes are involved in different aspects of cellular DNA damage response, activated as a result of exposure to DNA cross-linking agents. One of the genes, named *FANCD1*, has been identified as *BRCA2* (reviewed in reference 8). Further investigation revealed interaction between the BRCA2 protein and a protein called PALB2 (Partner And Localizer of BRCA 2). Some breast cancer families show mutations in the *PALB2* gene, suggesting that this is a low penetrance breast cancer predisposing gene (8). Another gene involved in FA, called *BRIP1*, was also classified as a low penetrance breast cancer predisposing gene (8). Taken together, these findings demonstrate an interesting link between two human genetic diseases (FA and familial breast cancer) but also reflect the importance of DNA damage response genes in both diseases.

Several more DNA damage response genes have been identified as low penetrance breast cancer predisposing genes. These include: *ATM*, *NBS1*, *CHEK2* and *RAD50* (8). ATM is a DNA damage signalling gene, affected in patients suffering from Ataxia telangiectasia (AT). The major symptom of AT is the sensitivity of patients to ionizing radiation and predisposition to cancer. *NBS1* is a gene affected in a human disease called Nijmegen breakage syndrome (NBS). Symptoms of this disease are very similar to AT. *CHEK2* is a gene encoding a kinase enzyme involved in DNA damage response. *RAD50* is involved in the repair of DNA double strand breaks through homologous recombination. In addition to these 6 low penetrance genes involved in DNA damage response, two more genes have been implicated in familial breast cancer. They are both tumor suppressor genes: *p53*, the function of which is to block cell cycle progression after genotoxic stress and *PTEN*, a gene that increases risk for breast, colon and thyroid cancer (8).

New low penetrance breast cancer predisposing genes

Given that *BRCA1/2* and low penetrance genes shown in Table 1 account for 50% of familial breast cancer cases, it is necessary to identify the remaining 50% genes involved in this disease. Classical genetic analysis, which relies on tracing patterns of inheritance in high-risk families, by using genetic markers inherited together with the disease gene, is limited in the case of low penetrance genes. A potential solution may be large genetic association studies. These studies analyze single genes in a group of people with a particular disease, relative to a group of healthy control people. The power of association studies can be enhanced by applying methods capable of simultaneously analyzing large number of genes/DNA sequences. Such methods have been developed or result directly from the human genome project (HGP).

One of the greatest achievements of the HGP was the identification of genetic variations, or variations in the DNA sequence, between individuals. The DNA sequence is determined by the distribution of four chemical building blocks of DNA, or DNA bases: adenine (A), thymine (T), guanine (G) and cytosine (C). The human genome, defined as the total human DNA sequence, contains three billion of these DNA bases. The DNA sequence in each individual is 99.9% the same. The remaining 0.01% of the human DNA sequence, or 1 in 1200 DNA bases, is different in each individual.

Let us assume a hypothetical DNA sequence on a human chromosome 16 to be: AGGTTCAGATCCT. This sequence is the same in all people, except for the underlined third base G, which can differ in different individuals. One person may have G on that position, whereas others may have A, T, or C bases at the same position. These DNA bases, at specific genomic locations, that differ between individuals, are called single nucleotide polymorphisms (SNPs; pronounced snips). SNPs constitute, by far, the greatest type of genetic variation and it has been estimated that the total number of common SNPs in the human genome is at least 10 million. Geneticists can use SNPs as markers to locate genes in DNA sequences. Let us assume that the SNP above (G in third position) could be associated with a risk for familial breast cancer. In order to test if this assumption is true, we can simply compare a large number of familial breast cancer cases with the same number of healthy individuals, and if we find that G dominates in breast cancer cases and T, or A, or C in healthy individuals, we can say that G, at that particular position, is definitely associated with breast cancer. Using the knowledge generated through HGP, we can then identify the genomic locus carrying the sequence with the above SNP. The identified locus may be within a specific gene, or in the vicinity of a specific gene. That particular gene is now assumed to be associated with breast cancer.

A scenario roughly similar to the above has been recently used to identify several new low penetrance genes, affected in familial breast cancer. A large research consortium, led by a research group from Cambridge University, used the most modern technology to determine or type a large number of SNPs in breast cancer families and healthy individuals, in a three-stage study (4). In the first stage, a panel of 266 722 SNPs were typed in 408 breast cancer cases and 400 healthy individuals. Statistical analysis revealed some SNPs associated with breast cancer cases. In the second stage, approximately 5% of the most significant SNPs (~12 000) were selected and typed in 3990 invasive breast cancers and 3916 control individuals. In the third stage, the 30 most significant SNPs were typed in ~ 22 000 breast cancer cases and a similar number of control individuals. This analysis identified 5 new loci exhibiting a significant association

Table 2 Recently identified (May 2007) low penetrance genes that confer breast cancer risk according to reference 4

Gene	Abbreviation	Function	Chromosome position
FGFR2	Fibroblast growth factor receptor 2	Receptor tyrosine kinase; Tumor suppressor	10q25.3-q26
TNRC9	Trinucleotide repeat containing 9	Transcription factor	16q12.1
MAP3K1	Mitogen activated protein 3 kinase 1	Kinase enzyme	5q11.2
LSP1	Lymphocyte specific protein 1	Regulates leukocyte recruitment to inflamed sites	11p15.5

with breast cancer. Four of these loci contain known genes including: *FGFR2*, *TNRC9*, *MAP3K1* and *LSP1*, which are now assumed to be low penetrance breast cancer genes (Table 2). The gene in the fifth locus has not yet been identified. The only gene that has previously been associated with breast cancer risk is *FGFR2*. This is a receptor tyrosine kinase which is amplified in 5-10% of breast tumors. The remaining three genes, *TNRC9*, *MAP3K1* and *LSP1*, have not been associated with breast cancer risk before.

This study is significant for several reasons. First, the four genes identified are not associated with DNA damage response (see Table 2 for their functions). This is in contrast to *BRCA1/2* and low penetrance genes from Table 1, almost all of which are involved in DNA damage response. This indicates a variety of molecular or genetic pathways that contribute to breast cancer. Second, the study adds further weight to the notion that breast cancer is essentially a polygenic disease in which each inherited gene adds a specific value of risk for breast cancer. Third, the study demonstrates the emerging power of a methodology resulting directly from HGP. It is now possible to apply the same approach to search for genes involved in other types of cancer or any other human disease.

Concluding remarks

Breast cancer is one of the best understood forms of human cancer at molecular and genetic levels. There are at least 14 genes known to be associated with breast cancer risk, two of which are high penetrance genes and the rest are low penetrance genes (Tables 1 and 2). Therefore, familial breast cancer is a polygenic disease (a disease caused by multiple genes). It remains unknown how many more low penetrance breast cancer predisposing genes exist, but it seems likely that future research will uncover new genes. The greatest breast cancer risk is conferred by high penetrance genes *BRCA1/2*. Low penetrance genes confer a much lower risk, which can increase by inheriting more than one of these genes.

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Acknowledge anyone who contributed towards the study by making substantial contributions to conception, design, acquisition of data, or analysis and interpretation of data, or who was involved in drafting the manuscript or revising it critically for important intellectual content, but who does not meet the criteria for authorship. List the source(s) of funding for the study, for each author, and for the manuscript preparation in the acknowledgements section.

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Sample References

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More than six authors:

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Organization as author:
Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. Hypertension. 2002;40(5):679-86.

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21st century heart solution may have a sting in the tail. BMJ. 2002;325(7357):184.

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Geraud G, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. Headache. 2002;42(Suppl 2): S93-9.

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Glauser TA. Integrating clinical trial data into clinical practice. Neurology. 2002;58(12 Suppl 7):S6-12.

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Banit DM, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. Clin Orthop. 2002;(401):230-8.

Letters or abstracts:
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Books and Other Monographs

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Chapter in a book:
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Conference paper:

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Tynan T. Medical improvements lower homicide rate: study sees drop in assault rate. The Washington Post. 2002 Aug 12;Sect. A:2 (col. 4).

Dictionary and similar references:

Dorland's illustrated medical dictionary. 29th ed. Philadelphia: W.B. Saunders; 2000. Filamin; p. 675.

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Foley KM, Gelband H, editors. Improving palliative care for cancer [monograph on the Internet]. Washington: National Academy Press; 2001 [cited 2002 Jul 9]. Available from: http://www.nap.edu/books/0309074029/html/.

Homepage/Web site:

Cancer-Pain.org [homepage on the Internet]. New York: Association of Cancer Online Resources, Inc.; c2000-01 [updated 2002 May 16; cited 2002 Jul 9]. Available from: http://www.cancer-pain.org/.

Part of a homepage/Web site:

American Medical Association [homepage on the Internet]. Chicago: The Association; c1995-2002 [updated 2001 Aug 23; cited 2002 Aug 12]. AMA Office of Group Practice Liaison; [about 2 screens]. Available from: http://www.ama-assn.org/ama/pub/category/1736.html

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