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Screening for Germline *p53* Mutations in Pediatric and Adult Patients of High-Risk Groups in Poland

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Abstract. Germline mutations of the *p53* gene lead to cell transformation in various tissues. Such a complex cancer phenotype makes it difficult to recognize the carriers of the defective allele. Several studies undertaken to identify high-risk groups found germline *p53* mutations in familial cancer aggregations and in patients with multiple tumors. We screened 189 pediatric and 48 adult patients. The high-risk groups comprised 41 patients with a family history of cancer and 35 with multiple neoplasms. Furthermore, 124 tumors were screened for somatic mutations. *p53* exons 2 to 11 were analyzed by polymerase chain reaction and single strand conformation polymorphism (PCR-SSCP) followed by direct sequencing of abnormal DNA fragments. No germline *p53* mutations were found and somatic mutations were detected in 5 of 59 sarcomas, globally, in 8 of 124 tumors. In conclusion, in Poland, *p53* alterations do not seem very important for the predisposition to malignancy and development of sarcomas.

Key words: *p53* tumor suppressor gene; germline mutation; Li-Fraumeni syndrome; Li-Fraumeni-like families; PCR-SSCP; direct sequencing.

Introduction

Inherited mutations of the *p53* tumor suppressor gene are associated with a complex cancer phenotype. Germline *p53* mutations had been discovered in patients with Li-Fraumeni syndrome (LFS)[21, 26]; however, they were also found in children and young adults with second malignant neoplasms[20] and in patients with sarcomas[29]. In the latter group, interestingly, some of the sarcoma patients diagnosed positive for the germline *p53* mutation appeared to have a family history of cancer.

Patients with LFS have strong familial histories of several tumor types, mostly bone and soft tissue sarco-

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mas, breast cancers, leukemias and brain tumors[12, 17, 19]. Recent studies have identified several other familial cancer aggregations associated with germline *p53* mutations which did not fulfill all the criteria for LFS and were described as Li-Fraumeni-like families (LFL)[5, 7, 24, 32]. As the number of LFS and LFL families has increased, the spectrum of *p53* associated cancers has become broader. However, some cancers have been observed specifically in families from countries which have a high incidence of sporadic cases, for instance gastric cancers, which have been predominantly found in Japanese families[15]. VARLEY et al.[31] compared the cancer phenotype in LFS and LFL families in relation to the presence or absence of a germline *p53* mutation and, although the number of families was small, showed that in the *p53*-positive families there was an excess of brain tumors (19% of all cancers vs 6% in the *p53*-negative families) and adrenocortical cancers (found only in families with *p53* mutations). In mutation-negative families, on the other hand, there appeared to be an excess of hematological neoplasms (10 vs 2%) and genitourinary system tumors (8 vs 2%).

The reported incidence of germline mutations of the *p53* gene in LFS families was as high as 50–70% and somewhat lower in LFL families[11, 15, 31]. For other at high-risk groups different figures have been published[8, 10, 16, 18, 22, 34]. However, it has been estimated that around 1% of breast cancer cases with an inherited component arise due to the *p53* mutation[4, 25].

The analysis of clinical data on cancer incidence in individuals with germline mutations of the *p53* gene shows that the type of cancer and the age at which disease manifests clinically are quite different even in family members carrying the same mutation. These observations suggest that some additional factors, e.g. genetic, environmental or life-style, are necessary to initiate cancer disease in mutant *p53* carriers. A genetic or functional defect responsible for the increased risk of cancer development in LFS/LFL families with no *p53* mutation found is yet to be discovered[3, 14, 23, 31]. Germline mutations of *CHK2* gene recently found in these families may appear one of such defects[2].

Our goal has been to identify LFS/LFL families and to learn of the potential contribution of germline mutations of the *p53* gene to childhood malignancies in Poland. Here we report the results of screening for germline *p53* mutations in groups of patients at high-risk of carrying the mutation: 1) in children or young adults with a family history of cancer and 2) in patients with multiple primary tumors, and also in pediatric patients with sporadic tumors, mainly those occurring in a cancer phenotype associated with germline *p53* mutations.

Materials and Methods

Patients and clinical samples. Blood and tumor samples were obtained from pediatric patients undergoing treatment at the Department of Pediatric Surgery and the Department of Pediatric Hematology and Oncology, Wrocław Medical University or at the Clinic of Pediatric Oncology, Institute of Mother and Child, Warsaw. Blood samples were also obtained from adult patients with multiple primary tumors or with a family cancer history treated at the Department of Oncology and Clinic of Gynecological Oncology, Wrocław Medical University, and Lower Silesia Oncological Center, Wrocław, or at the Out-patients' Department of Oncology and Chemotherapy, ZOZ, Świdnica. Information concerning the family history of cancer was obtained in a direct interview. Informed consent was obtained from participating patients or their parents.

Tumor specimens from pediatric patients were obtained at surgical resections, flash frozen and stored at –80°C until DNA extraction. These samples were collected as extra diagnostic materials for use in molecular analysis. Blood samples of 100 healthy persons and cell lines with known *p53* gene alterations, HL-60, RAJI, HT29, LS180 and AF1 (LFS fibroblasts kindly supplied by Dr. M. B. Kastan), were used as controls.

Polymerase chain reaction and single-strand conformation polymorphism (PCR-SSCP) analysis and direct DNA sequencing. DNA from blood and tumor samples was prepared according to standard protocols with proteinase K digestion and phenol/chloroform extraction. The strategy used to screen for mutations of the *p53* gene was based on a PCR amplification of exons 2 to 11 from genomic DNA and SSCP analysis of the products[30]. A separate pair of primers was used to amplify each exon except for exon 5, in which two partially overlapping PCR fragments were studied. The reaction mixtures contained 50–100 ng DNA, 1×PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$), 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM TTP, 0.04 mM dCTP, 1 μM of each primer, 0.4 U Taq polymerase (Perkin-Elmer) and 1.0 μCi (α-^{32}P)-dCTP (3000 Ci/mmol, Du Pont NEN) in a final volume of 20 μl. PCR products were generated in a Biometra thermal cycler in 35 cycles using the following thermal profile: 94ºC for 30 s, 55 to 58ºC for 30 s, and 70ºC for 30 s. An aliquot of the reaction mixture was withdrawn and diluted with a loading buffer containig 95% (v/v) formamide, 20 mM EDTA, 0.05% (w/v) xylene cyanole and 0.05% (w/v) bromophenol blue. The resulting mixture in a volume of 2–5 μl was denatured at 85ºC for 5 min and applied to 6% nondenaturing polyacrylamide

gels (49 : 1 ratio of acrylamide to bisacrylamide) with and without 10% glycerol. Electrophoresis was performed with a constant power of 8–10 W, at room temperature or at 8ºC. Autoradiography was carried out at –80ºC for 10–24 h, with an intensifying screen. Abnormal DNA fragments detected by SSCP were further analyzed by direct cycle sequencing with the ABI Prism™ dye terminator cycle sequencing kit (Perkin-Elmer) on the ABI 373A DNA Sequencer (Perkin-Elmer). Polymorphism in the PCR fragment from exon 6 (codon 213) was also verified by TaqI restriction followed by polyacrylamide gel electrophoresis[6].

Results

The analysis of the *p53* gene was performed in 189 pediatric and 48 adult patients. Groups of patients at high-risk of carrying the germline mutation comprised 41 patients with a family history of cancer: 28 children and 13 young adults (aged ≤ 50 years) selected on the basis of the clustering of two or more tumors (Table 1), and 35 adults with multiple primary tumors. Eleven of the families listed in Table 1 were considered LFL families because of the occurrence of the following conditions: 1) proband with any childhood tumor or sarcoma, brain tumor, breast or adrenocortical cancer diagnosed at ≤50 years, 2) one other case of cancer characteristic of LFS affecting a first or second degree relative or a first cousin under the age of 50 years, or two invasive cancers in relatives. In the group of patients with multiple tumors, the ages ranged from 33 to 73 years (mean 52.7; median 51) at the time of the initial diagnosis, and they were from 36 to 75 years old (mean 57.4; median 58) when the second tumor was found. The patients' age, the histologic features of the tumors and the times to the development of the second cancer differed widely. This heterogeneity is suggestive of a random acquisition of genetic alterations which led to cancer. Clinical data for all pediatric patients (familial and sporadic cases) are summarized in Table 2. In the studied group there were 89 (47%) children with bone tumors or soft tissue sarcomas, the rest comprising children with various hematological malignancies, germinal, kidney, liver and thyroid tumors, as well as with neuroblastoma.

SSCP analysis and sequencing of constitutional DNAs of our patients, besides the known polymorphisms in exon 4, 6 and intron 2, showed no alterations in exons 2 to 11 of the *p53* gene. Differences in migration patterns of PCR fragments produced with control DNA samples with wild or mutated *p53* were readilly distinguished. Figure 1 shows an example of the SSCP analysis of exon 7 and 8, including the most frequent alterations in codons 248 and 273. The polymorphism in exon 6 has been estimated to occur in 3.5% of the population[6], and our data – 3.85% (5 individuals of 130) is consistent with this.

In parallel, the *p53* gene was examined in a series of 124 pediatric tumors. SSCP and sequencing of PCR

Table 1. Families with cancer history screened for germline *p53* mutations

Probands		Number of tumors/affected relatives	Cancers in relatives	Number of tumors/number of LFL families
diagnosis (range of age in years)	number			
Rhabdomyosarcoma (4–14)	6	7/7	leukemia, Wilms, colon, lip, liver, lung, uterus	6/2
Mesenchymoma (2,13)	2	2/2	breast, larynx	
Fibrosarcoma (19, [brain, 26], 33)	2	4/4	colon, lung, uterus	7/2
Ewings sarcoma (1 month–14)	4	9/9	brain, breast, colon, lip, lung, uterus	10/3
Lymphoma (6–17)	4	12/11	brain, breast, colon, gynecological lesion, liver, kidney, stomach	8/1
Neuroblastoma (1, 4)	2	2/2	breast, stomach	
Wilms (2–8)	3	9/6	leukemia, neuroblastoma, Wilms, larynx, lip, lung, stomach, uterus	7/1
Breast (35–50)	8	11/11	breast, colon, gynecological lesion, ovary, stomach, NF1	4/1
Miscellaneous (3–50): neurofibroma (NF1), melanoma, dysgerminoma, hepatoblastoma, lymphogranuloma, kidney rhabdoid tumor, adrenocortical cancer, thyroid, pancreas, breast, colon, uterus	10	16/15	fibromas, larynx polyposis, papilloma, breast, cervix, jaw, lip, lung, pancreas, ovary, skin, stomach, uterus	6/1
Total	41	72/67		48/11

NF1 – neurofibromatosis type 1.

Table 2. Pediatric patients screened for germline *p53* mutations

Tumor origin and diagnosis	Number of patients	Total	
		n	%
Bone		41	21.7
osteosarcoma	15		
Ewings sarcoma	16		
other	10		
Soft tissue		48	25.4
myogenic	15		
malignant fibrous histiocytoma	2		
malignant fibrous lesions	4		
malignant neurogenic tumors	7		
other	20		
Neuroblastoma		20	10.6
Hematological		26	13.8
lymphoma	17		
other	9		
Germ cell tumors		18	9.5
teratoma	13		
Renal		21	11.1
Wilms tumor	19		
Other		15	7.9
hepatoblastoma	8		
Total		189	100

products identified 8 mutations (Table 3): 2 of 13 osteosarcomas, 1 of 2 malignant fibrous histiocytomas (MFH), 1 of 2 aggresive fibromatoses, 1 of 11 lymphomas, 1 leukemia, 1 hemangioendothelioma and 1 of 4 hepatoblastomas. Unfortunately, blood samples of osteosarcoma patients could not be obtained to determine whether the mutation was of somatic or germline origin; the SSCP analysis of blood DNA of other patients once again demonstrated the wild pattern, indicating that the *p53* mutations were indeed of somatic origin.

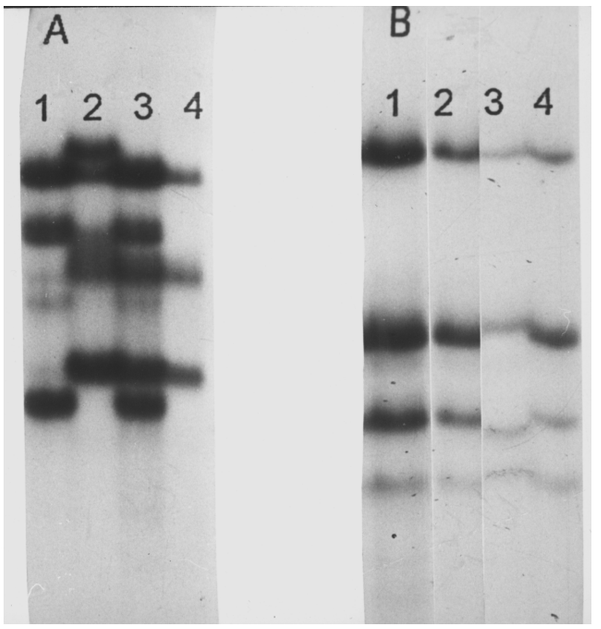

Fig. 1. SSCP analysis of exons 7 and 8 of the *p53* gene in pediatric tumor samples and cell lines. A – exon 7: lane 1 – IMD375 osteosarcoma; lane 2 – IMD374 osteosarcoma; lane 3 – AF1 fibroblasts; lane 4 – normal DNA. B – Exon 8: lane 1 – LS180 colon carcinoma; lane 2 – LS180 selectant – 5W; lane 3 – HT29 colon carcinoma; lane 4 – normal DNA. A shift in the ssDNA pattern was observed in samples: IMD375, IMD374, AF1 and HT29

Discussion

Intensive studies on germline *p53* mutations have been performed in only a few countries. Therefore, the available data are insufficient to provide reliable assessment of a contribution of constitutional alterations of the *p53* gene to hereditary cancers and to estimate their frequency and penetrance[5, 8, 11, 15, 31].

Table 3. *p53* gene alterations detected in pediatric malignancies

Sample	Tumor or cell line	Exon	Codon	Change	Amino acid substitution
IMD374	Osteosarcoma	7	237	ATG→ATA	Met→Ile
IMD375	Osteosarcoma	7	248	CGG→CAG	Arg→Gln
PW30	MFH	8	282	CGG→TGG	Arg→Trp
PW23	Aggressive fibromatosis	5	175	CGC→CAC	Arg→His
PW69	Hemangioendothelioma	7	248	CGG→CAG	Arg→Gln
PW153	Leukemia	9	330	CTT→CGT	Leu→Arg
PW116	Lymphoma	5	177	19 bp deletion	Frame-shift
PW134	Hepatoblastoma	5	185	AGC→AAC	Ser→Asn
PW184	Ovary carcinoma	6	213	CGA→CGG/A	Polymorphism
Positive controls	HT29 colon carcinoma	8	273	CGT→CAT	Arg→His
	AF1 fibroblasts	7	248	CGG→CAG	Arg→Gln

IMD – Institute of Mother and Child in Warsaw.
PW – Wrocław Medical University.
MFH – malignant fibrous histiocytoma (material from recurrence).

Analysis of current data on germline *p53* mutations revealed that around one eighth of *p53*-positive families was identified on the basis of just one tumor, and in another one eighth only one generation was studied[15]. On the other hand, even in individuals from a single family, thus carrying the same mutation, the age at which disease manifested clinically varied significantly. Moreover, in a significant proportion of LFS/LFL families, no *p53* mutation was found[11, 23, 32]. These observations, as well as the comparison of the cancer phenotype in LFS/LFL families with trends in the general population, point to other still unidentified cancer genes or factors, e.g. genetic, environmental or related to life-style, which influence the manifestation of the disease or actually cause it[2, 13, 14]. At present, it is impossible to assess the weight of each of the factors[28].

In this investigation of the incidence of germline *p53* mutations in the Polish population, we decided to screen patients of an assumed genetic predisposition, i.e., children and young adults (≤50 years) with a family cancer history, patients with multiple tumors, as well as pediatric patients with sporadic tumors. The high-risk groups comprised 41 patients with a family history of cancer and 35 adults with multiple primary tumors. Patients with a family history of cancer were selected on the basis of the clustering of two or more tumors. Such relaxed criteria for patient selection were established as we did not want to restrict the spectrum of studied cancer phenotypes, specially in view of the fact that the *p53* associated phenotype has not been well defined and might differ significantly between different ethnic and geographical populations. Analysis of exons 2–11 in all these cases did not reveal any germline *p53* mutation. The negative result of our search might be random due to the number of patients in high-risk groups or might reflect the criteria used to select patients for the study. Another possible explanation of the achieved result is the sensitivity of the SSCP assay, though this hardly to have had a critical effect on our data, because our controls, the most frequent mutations at codons 248 and 273, are easily distinguished from wild sequence. Thus, the negative result of our search most likely reflects a very low incidence of constitional *p53* mutations in Poland. It seems possible that, due to the complex cancer phenotype in mutant-*p53* carriers, access to health care and cancer directed treatments may indirectly affect the occurence of germline *p53* mutations in a population.

Nevertheless, even if, in some of our families cancer aggregations have occurred by chance, in most families a high-risk of cancer is likely to be associated with other cancer genes[2] or genes coding for carcinogens detoxifying enzymes, mutations or specific alleles of which might determine a significant inherited predisposition to diverse types of cancers[14]. As the alleles of a gene family (e.g. GST genes) segregate independently, the inherited predisposition might broadly differ in family members[13].

The number of somatic *p53* mutations detected in our series of common pediatric malignancies was low (8 of 124 tumor samples). However, this result is consistent with previous mutational studies[1, 9, 10, 27, 30, 33]. WÜRL et al.[35], for instance, detected only 6 mutations in 35 (17%) myogenic sarcomas and none in 34 fibrosarcomas and 31 malignant neural tumors.

Taken together, our search for germline *p53* mutations comprised both cancer patients of high-risk groups and a significant proportion of new onsets of bone and soft tissue sarcomas in Poland in the period of 1994–1997. As the number of studied patients was considerable and SSCP analysis detected the most frequent *p53* mutations, it seems reasonable to accept that germline *p53* mutations in the Polish population are indeed rare.

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