Antioxidant defence-related genetic variants are not associated with higher risk of secondary thyroid cancer after treatment of malignancy in childhood or adolescence

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Background. Thyroid cancer is one of the most common secondary cancers after treatment of malignancy in childhood or adolescence. Thyroid gland is very sensitive to the carcinogenic effect of ionizing radiation, especially in children. Imbalance between pro- and anti-oxidant factors may play a role in thyroid carcinogenesis. Our study aimed to assess the relationship between genetic variability of antioxidant defence-related genes and the risk of secondary thyroid cancer after treatment of malignancy in childhood or adolescence.

Patients and methods. In a retrospective study, we compared patients with childhood or adolescence primary malignancy between 1960 and 2006 that developed a secondary thyroid cancer (cases) with patients (controls), with the same primary malignancy but did not develop any secondary cancer. They were matched for age, gender, primary diagnosis and treatment (especially radiotherapy) of primary malignancy. They were all genotyped for *SOD2* p.Ala16Val, *CAT* c.-262C>T, *GPX1* p.Pro200Leu, *GSTP1* p.Ile105Val, *GSTP1* p.Ala114Val and *GSTM1* and *GSTT1* deletions. The influence of polymorphisms on occurrence of secondary cancer was examined by McNemar test and Cox proportional hazards model.

Results. Between 1960 and 2006 a total of 2641 patients were diagnosed with primary malignancy before the age of 21 years in Slovenia. Among them 155 developed a secondary cancer, 28 of which were secondary thyroid cancers. No significant differences in the genotype frequency distribution were observed between cases and controls. Additionally we observed no significant influence of investigated polymorphisms on time to the development of secondary thyroid cancer.

Conclusions. We observed no association of polymorphisms in antioxidant genes with the risk for secondary thyroid cancer after treatment of malignancy in childhood or adolescence. However, thyroid cancer is one of the most common secondary cancers in patients treated for malignancy in childhood or adolescence and the lifelong follow up of these patients is of utmost importance.

Key words: secondary thyroid cancer; antioxidant genes; genetic polymorphism

Introduction

Modern treatment modalities and better diagnostic techniques greatly improved the survival of children and adolescents with malignancies.[1,2] With increasing number of survivors and years of follow up late effects of treatment are encountered more frequently.[3,4]

The most detrimental late effects are secondary cancers. Several studies report on increased risk of subsequent secondary cancers even several decades after treatment of primary malignancy.[5] Increased incidence of secondary thyroid cancer was reported even up to 40 years after radiotherapy.[6-9]

The thyroid gland is very sensitive to the carcinogenetic effect of ionizing radiation, especially in children.[7] Ionizing radiation damages DNA directly or indirectly through production of free radicals and reactive oxygen species (ROS). It has been shown that gamma radiation and hydrogen peroxide (H_2O_2), which is one of the ROS, induce similar DNA damages in the thyroid.[10] Oxidative DNA damage involves single- or double DNA strand breaks, purine and pyrimidine or deoxyribose modifications as well as DNA cross links. ROS can also damage the cell through lipid peroxidation, protein modification, membrane disruption and mitochondrial damage.[11,12]

The thyroid cell is constantly exposed to ROS and an imbalance between pro- and anti-oxidative factors has been suggested as an important mechanism in thyroid carcinogenesis. The accumulation of oxidative DNA damage may drive genomic instability events and lead to somatic mutations. Many studies have shown that oxidants are increased and antioxidants are decreased in patients with thyroid cancer.[13-19]

The most important antioxidants in the thyroid are antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT). Manganese superoxide dismutase (SOD2) is the major antioxidant in mitochondria, catalysing the dismutation of superoxide anion to H_2O_2, which is then reduced to water by CAT or GPX.[20,21] Many studies have investigated genetic variability in genes coding for antioxidant enzymes and their relationship to cancer risk, however the results were inconclusive[22] and the data on thyroid cancer risk are lacking.[23]

The most common polymorphism in the gene coding for SOD2 (SOD2) leads to substitution of alanine (Ala) with valine (Val) at codon 16 (p.Ala16Val) and affects transport of the enzyme into the mitochondria.[24] According to several studies the 16Ala allele of SOD2 polymorphism is associated with an increased risk of prostate and oesophageal cancer.[21,25]

CAT activity is affected by functional single nucleotide polymorphism (SNP) CAT c.-262C>T in the promoter region of CAT gene, which leads to lower catalase activity. This polymorphism was implicated in increased susceptibility to breast and cervical cancer.[26,27]

The most common GPX1 polymorphism results in the amino acid substitution of leucine with proline at codon 200 (p.Leu200Pro) and results in lower enzyme activation. As it may influence the balance between oxidative stress and antioxidant defence, it may therefore increase cancer risk.[28] Indeed several studies associate GPX1 p.Pro200Leu polymorphism with increased susceptibility to prostate and breast cancer.[29,30]

Glutathione S-transferase (GSTs) enzymes, encoded by GST genes, are implicated in detoxification of xenobiotics and reactive products of ROS, so they may have a crucial role in protecting tissue from oxidative damage.[31] Deletions of the GSTM1 and GSTT1 genes result in null genotypes and lead to impaired enzyme activity.[32] In GSTP1 two frequent SNPs resulting in an amino acid substitution have been reported. The GSTP1 p.Ile105Val SNP results in Ile to Val substitution near the active site and leads to decreased enzyme activity. The functional role of the GSTP1 p.Ala114Val polymorphism is not clear, however, reduced conjugation capacity was reported in the enzyme with both polymorphisms present.[30] Variants of these loci have been implicated in the aetiology of numerous cancers.[29,31-34]

There is some inconclusive data on GST polymorphisms in association to thyroid cancer risk. According to some studies individuals with homozygous deletions of GSTM1 or GSTT1 have an increased risk of thyroid cancer, whereas Lemos et al. found the opposite in his study.[33-35] Mertens et al. studied radiotherapy related malignancies in survivors of Hodgkin disease and found that individuals lacking GSTM1 but not GSTT1 were at increased risk of any subsequent SMN.[36] To our knowledge data on genetic variability of antioxidant enzymes in primary thyroid cancer are scarce, while no data have been published regarding the secondary thyroid cancer.

The aim of the present study was to investigate the relationship between genetic variability in antioxidant defence-related genes (SOD2 p.Ala16Val, CAT c.-262C>T, GPX1 p.Pro200Leu, GSTM1 deletion, GSTT1 deletion, GSTP1 p.Ile105Val and GSTP1 p.Ala114Val) and the risk of secondary thyroid cancer after treatment of malignancy in childhood or adolescence.

Patients and methods

Patients

A population based study of all patients known to have developed a secondary thyroid cancer after treatment of malignancy in childhood or adolescence was performed. A retrospective matched case-control study was designed. Individuals were eligible for inclusion in the study patients group (cases) if they were diagnosed with any kind of primary malignancy between 1960 and 2006 and before the age of 21 and were treated at the Department of Hematology and Oncology, University Children's Hospital, Ljubljana, or at the Institute of Oncology, Ljubljana and had a secondary thyroid cancer diagnosed 5 years or later after the primary malignancy. Study controls were patients with a primary malignancy in childhood or adolescence but free of secondary thyroid cancer. They were selected with a ratio of 1 control to 1 case matched for: type of the primary malignancy, treatment of primary malignancy, especially regarding the radiotherapy to the neck, head or mediastinal region, sex and age at the time of primary malignancy diagnosis (if possible not more than 2 years younger or older then the case). Study patients and study controls were identified from a search from the Cancer Registry of Slovenia.[37]

All patients with primary childhood or adolescent malignancy were followed up at the Department of Hematology and Oncology, University Children's Hospital, Ljubljana, or at the out-patient Clinic for Late Effects at the Institute of Oncology, Ljubljana.[38]

Thyroid follow-up included yearly thyroid stimulating hormone (TSH) and thyroglobulin level evaluation and occasional neck ultrasound. All patients with palpable nodules and/or elevated thyroglobulin levels underwent a neck ultrasound as a method commonly used in the work-up of thyroid diseases.[39] If malignancy was suspected, fine needle aspiration biopsy (FNAB) was performed. When papillary/follicular lesions were detected or were just suspected by cytology, thyroidectomy was performed at the Institute of Oncology, Ljubljana.

The follow up interval was defined as the time between primary malignancy and secondary thyroid cancer in the study group or between primary malignancy and the last appointment at the Out-Patient Clinic for Late Effects in the control group. All patient's data (demographic, clinical and treatment data) were collected from the patient's medical records.

Single experienced pathologist reviewed all the primary malignancies and corresponding thyroid cancers.

The study was approved by the Slovenian Ethics Committee for Research in Medicine (No.138/04/10) and was carried out according to the Declaration of Helsinki.

DNA isolation and genotyping

According to the presence of tumour or normal tissue on hematoxylin and eosin (HE) slides the pathologist chose one representative paraffin block from each biopsy and marked the tumour and normal tissue area on the block. From the marked area (if possible we chose normal tissue) two to three cores of 1 mm in diameter were obtained for DNA extraction using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.[40]

For two control patients genomic DNA was isolated from archived cytological smears of bone marrow specimens using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany).[41] For all other patients DNA was obtained from paraffin blocks contained tumour or normal tissue as described above.

Genotypes of *GPX1* p.Pro200Leu (rs1050450) and *SOD2* p.Val16Ala (rs4880) were determined by TaqMan genotyping method (Applied Biosystems, Foster City, CA, USA) as described previously.[42] Genotyping of *CAT* c.-262C>T (rs1001179), *GSTP1* p.Ile105Val (rs1695) and *GSTP1* p.Ala114Val (rs1138272) was carried out using a fluorescence-based competitive allele-specific (KASPar) assay (KBiosciences, Herts, UK).[43]

Multiplex polymerase chain reaction (PCR) was used for detection of *GSTM1* and *GSTT1* gene deletions. *GSTM1*, *GSTT1*, and *BGLO* genes were simultaneously amplified in a multiplex PCR reaction as previously described.[41] This approach allowed us to identify homozygous *GSTM1* or *GSTT1* gene deletion, but we could not distinguish between carriers of one or two copies of each gene.

Statistical analysis

Frequencies were used to describe the distribution of categorical variables and median and interquartile ranges were used for continuous variables. Standard chi-square test was used to assess deviation from Hardy-Weinberg equilibrium (HWE), comparing the distribution of genotype frequencies in the control group with the expected distribution within the population.[44]

To compare the genotype distribution, McNemar test for the analysis of matched samples based on binomial distribution was used. The influence of polymorphisms on the time to the occurrence of second cancer was examined by Cox proportional hazards model with stratification on the matched pairs to calculate relative risks (RRs) and their 95% confidence intervals (CIs).

All statistical analyses were carried out by IBM SPSS Statistics, version 19.0 (IBM Corporation, Armonk, NY, USA), except for odds ratios (OR) and 95% CIs in McNemar test that were calculated using GraphPad Software. A dominant genetic model was used in all statistical analyses and p values below 0.05 were considered statistically significant.

Results

Patients' characteristics

Based on data from The Cancer Registry of Slovenia, in the period between 1960 and 2006 a total of 2641 patients were diagnosed with primary cancer before the age of 21 years.[40] Among them 155 developed secondary cancer (5.9%), out of which 28 (18.1%) were secondary thyroid cancers.

Only 24 (85.7%) eligible cases were included in the study because we could not get the histopathological material for 4 controls. Therefore the study group included 8 (33.3%) males and 16 (66.7%) females with a median age of 12.7 years at diagnosis of primary cancer (range 7.1–6.7 years) and 29.2 years at diagnosis of secondary thyroid cancer (range 23.5–35.4 years). Six out of 24 patients (25.0%) were under 5 years old at the time of primary diagnosis. The control group included 8 (33.3%) males and 16 (66.7%) females with a median age of 12.9 years at diagnosis of primary cancer (range 5.0–15.4 years).

The most frequent primary cancer was Hodgkin's disease (HD) (15 pairs, 62.5%), then acute lymphoblastic leukemia (ALL) (2 pairs, 8.3%) and central nervous system (CNS) tumours (2 pairs, 8.3%). Non-Hodgkin lymphoma (NHL), neuroblastoma, rhabdomyosarcoma, nasopharyngeal carcinoma and ovarian tumours were observed in 1 pair each (4.2%). Most of the patients with secondary thyroid cancer received radiation therapy to the head, neck or mediastinum during the treatment for primary cancer (23 patients, 95.8%): 15 (62.5%) to the neck, 6 (25.0%) to the head and 2 (8.3%) to the mediastinum. The same distribution of irradiated sites was observed also in the control group.

The most frequent histology of secondary thyroid cancer was papillary carcinoma (23, 95.8%).

Only 1 tumour (4.2%) was follicular neoplasm of undefined malignant potential. Using TNM classification for staging most of thyroid cancer were stage 1 with tumour localised to the thyroid and/or lymph nodes (23, 95.8%), 1 (4.2%) was stage 2 with lung metastasis (M1). Among 12 (50.0%) T1 tumours (tumour diameter ≤ 2cm), there were 9 (37.5%) microcarcinoma (T1a: tumour ≤ 10 mm). There were 2 (8.3%) T2 tumours (tumour > 2 cm but ≤ 4 cm in greatest dimension, limited to the thyroid); 6 (25.0%) were T3 (minimal extrathyroid extension) and 3 (12.5%) were T4 (extending beyond the thyroid capsule to invade subcutaneous soft tissues, larynx, trachea, oesophagus, or recurrent laryngeal nerve) and 11 (45.8%) had regional lymph node metastasis (N1).

A total or near total thyroidectomy was carried out in all cases. Additional radioiodine treatment was applied to 19 (79.2%) patients. In 6 (25.0%) cases lymph node metastases were excised.

The follow up interval was comparable in both groups and was 19.6 (range 9.0–23.6) years in the primary group and 18.8 (range 12.80–27.6) years in the control group. Both groups did not differ significantly regarding the demographic data.

Genotype frequencies of the antioxidant defence-related genes are presented in the Table 1. All the investigated polymorphisms were in HWE in the control group.

To assess if the investigated polymorphisms influence the risk of secondary thyroid cancer, we performed a matched analysis. When all the cases were compared to controls, no significant differences in the genotype frequency distribution were observed (Table 2). There were also no differences in genotype distribution between microcarcinoma and other secondary thyroid cancers.

We also assessed the influence of investigated polymorphisms on time to development of secondary thyroid cancer using Cox regression with stratification on matched pairs. We have not observed any association between studied polymorphisms and the time pattern of occurrence of secondary thyroid cancers after treatment of malignancy in childhood or adolescence.

Discussion

In the present study we investigated if *SOD2* p.Ala16Val, *CAT* c.-262C>T, *GPX1* p.Pro200Leu, *GSTM1, GSTT1, GSTP1* p.Ile105Val and *GSTP1* p.Ala114Val polymorphisms influence the risk of secondary thyroid cancer after treatment of malig-

TABLE 1. Genotype frequencies of the antioxidant defence-related genes

SNP	Genotype	All patients N (%)	Cases N (%)	Controls N (%)	P_{HWE} controls
GPX1 rs1050450 p. Pro200Leu	CC	28 (58.3)	16 (66.7)	12 (50)	0.967
	CT	17 (35.4)	7 (29.2)	10 (41.7)	
	TT	3 (6.3)	1 (4.2)	2 (8.3)	
SOD2[a] rs4880 p.Val16Ala	GG	10 (21.7)	4 (16.7)	6 (27.3)	0.338
	GA	31 (67.4)	18 (75)	13 (59.1)	
	AA	5 (10.9)	2 (8.3)	3 (13.6)	
CAT rs1001179 c.-262G>A	GG	32 (66.7)	16 (66.7)	16 (66.7)	0.834
	GA	14 (29.2)	7 (29.2)	7 (29.2)	
	AA	2 (4.2)	1 (4.2)	1 (4.2)	
GSTP1 rs1695 p.Ile105Val	AA	22 (45.8)	10 (41.7)	12 (50)	0.432
	AG	23 (47.9)	12 (50)	11 (45.8)	
	GG	3 (6.3)	2 (8.3)	1 (4.2)	
GSTP1 rs1138272 p.Ala114Val	CC	39 (81.3)	19 (79.2)	20 (83.3)	0.106
	CT	8 (16.7)	5 (20.8)	3 (12.5)	
	TT	1 (2.1)	/	1 (4.2)	
GSTM1[b] gene deletion	non-null	23 (48.9)	14 (58.3)	9 (39.1)	
	null	24 (51.1)	10 (41.7)	14 (60.9)	
GSTT1[b] gene deletion	non-null	39 (83)	19 (79.2)	20 (87)	
	null	8 (17)	5 (20.8)	3 (13)	

CAT = catalase; GPX = glutathione peroxidase; GSTM1 = glutathione S-transferase Mu 1; GSTP1= glutathione S-transferase pi gene; GSTT1 = glutathione S-transferase theta 1; HWE= Hardy-Weinberg equilibrium; N = number; SNP = single nucleotide polymorphism; SOD2 = manganese superoxide dismutase; [a]data missing for 2 controls; [b]data missing for 1 control

nancy in childhood or adolescence. To the best of our knowledge no such study was yet performed in the secondary thyroid cancer, while data on the role of common functional polymorphisms in antioxidant defence-related genes in the primary thyroid cancer are scarce.[29]

In total 5.9% of patients diagnosed between 1960 and 2006 with primary malignancy before the age of 21 years has developed a secondary cancer. Secondary thyroid cancers represented 18% of secondary cancers. Our data are comparable to other studies and show that thyroid cancer is one of the most common secondary cancers after treatment of malignancy in childhood or adolescence. Similar to other studies the most frequent primary malignancy was Hodgkin's lymphoma and the median time to develop a secondary thyroid cancer was 19.6 years.[2-6]

According to several studies the SOD2 16Ala allele was associated with an increased risk of prostate and oesophageal cancer, but with decreased risk of lung cancer.[21,24] Never the less, the meta-analysis showed no significant effect of SOD2

TABLE 2. Influence of selected polymorphisms on the risk for secondary thyroid cancer

SNP	OR (95% CI)	p
GPX1 (rs1050450)	0.43 (0.07-1.88)	0.344
SOD2[a] (rs4880)	1.50 (0.36-7.23)	0.754
CAT (rs1001179)	1.00 (0.27-3.74)	1.000
GSTP1 (rs1695)	1.40 (0.38-5.59)	0.774
GSTP1 (rs1138272)	1.25 (0.27-6.30)	1.000
GSTM1 (gene deletion)[b]	0.43 (0.07-1.88)	0.344
GSTT1 (gene deletion)[b]	2.00 (0.29-22.11)	0.687

CAT = catalase; CI = confident interval; GPX = glutathione peroxidase; GSTM1 = glutathione S-transferase Mu 1; GSTP1= glutathione S-transferase pi gene; GSTT1 = glutathione S-transferase theta 1; OR = odd ratio; SNP = single nucleotide polymorphism; SOD2 = manganese superoxide dismutase; [a]data missing for 2 controls; [b]data missing for 1 control

p.Val16Ala polymorphism on overall cancer risk.[21] This is also in concordance with our data that show no association between the SOD2 polymorphism and the risk of secondary thyroid cancer.

Recent studies suggested that the *GPX1* p.Pro200Leu polymorphism increased the susceptibility to bladder cancer[27], whereas a meta-analysis showed no significant association of *GPX1* p.Pro200Leu polymorphism with cancer risk in general.[45] Similar to the meta-analysis we observed no association between *GPX1* p.Pro200Leu polymorphism and the risk of secondary thyroid cancer.

CAT polymorphism was implicated in cancerogenesis of several tumours, including breast and cervical cancer[24], but again, meta-analysis has not confirmed these observations for the breast cancer risk.[46] Our results also showed no association between *CAT* polymorphism and risk of secondary thyroid cancer.

Genetic variability at the *GSTM1, GSTT1 and GSTP1* loci has been linked to increased susceptibility to several cancers, including thyroid cancer.[28,29,31] Our study was the first to analyse the association between the *GST* polymorphisms (*GSTM1, GSTT1, GSTP1* p.Ile105Val and *GSTP1* p.Ala114Val) and the risk of developing secondary thyroid cancer after treatment of malignancy in childhood or adolescence, however, we were not able to detect any significant association. Mertens *et al.* found only a non-significantly increased risk of thyroid cancer in *GSTM1* or *GSTT1* homozygous patients that had Hodgkin lymphoma as a primary cancer[36], but a meta-analysis concluded that GST polymorphisms are unlikely to be major determinants of susceptibility to primary thyroid cancer.[29]

Our results are comparable to the results of relevant studies on antioxidant defence-related polymorphisms and the risk of cancer in general. While the sample size is small and therefore in some instances lacks statistical power, its prime advantage is the homogeneity of data because we designed a population-based study, which included all patients diagnosed and treated for secondary thyroid cancer in Slovenia after treatment of malignancy in childhood or adolescence between 1960 and 2006.

In conclusion, we observed no association of common functional polymorphisms in antioxidant defence related genes with the risk for secondary thyroid cancer after treatment of malignancy in childhood or adolescence. However, thyroid cancer is one of the most common secondary cancers after treatment of malignancy in childhood or adolescence and it can develop several decades after the treatment. Hence the lifelong follow up of patients with childhood or adolescent malignancy is of utmost importance and further studies on genetic factors associated with thyroid cancer risk should be performed.

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