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HEAD AND NECK

The role of genetic factor in etiopathogenesis of squamous cell carcinoma of the head and neck in young adults

Wojciech Gawęcki · Magdalena Kostrzewska-Poczekaj · Marzena Gajęcka · Piotr Milecki · Krzysztof Szyfter · Witold Szyfter

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Abstract Patients with squamous cell carcinoma of the head and neck (HNSCC) aged less than 45 years are categorized as young adults (YA) and in opinion of many authors in comparison to older (typical) patients (OP) are characterized with more serious form of the disease and often lack the classical risk factors associated with the illness. Hence, there is a need of an exact clinical analysis and a search for additional causative factors. The purpose of this study was to estimate the role of genetic factors in the etiology of HNSCC in YA. Studies carried out on 60 patients of the study group (YA) and 72 older control patients were directed to: (1) a degree of spontaneous and induced chromosome breaks estimated by bleomycin test, (2) a degree of spontaneous and induced DNA damage and a potential of DNA repair determined by comet assay and (3) polymorphism of genes of glutathione transferases *M1* and *T1*, responsible for detoxification of metabolites of carcinogens of tobacco smoke, studied by PCR-based genotyping. The level of chromosome breaks (spontaneous and induced), the level of DNA damage (spontaneous and induced), DNA repair potential and the distribution of polymorphic variants of *GSTT1* gene are not significantly different in YA and in OP, which suggests that these factors do not appear the causative factors for HNSCC in young age. The significant risk factor of HNSCC in YA may be *GSTM1* null genotype, which may cause the defective detoxification of metabolites of polycyclic aromatic hydrocarbons of tobacco smoke.

Keywords Squamous cell carcinoma of the head and neck · Young adults · Genetic factor

W. Gawęcki (✉) · K. Szyfter · W. Szyfter
Department of Otolaryngology and Laryngological Oncology,
Karol Marcinkowski University of Medical Sciences,
Przybyszewskiego 49, 60-355 Poznan, Poland
e-mail: wojgaw@interia.pl

M. Kostrzewska-Poczekaj · M. Gajęcka · K. Szyfter
Institute of Human Genetics,
Polish Academy of Sciences, Poznan, Poland

P. Milecki
Radiotherapy Department,
Great Poland Cancer Centre, Poznan, Poland

Introduction

Squamous cell carcinoma of the head and neck (HNSCC) is a malignant tumor arising most frequently in non-keratinized epithelial tissue of the upper part of the respiratory or gastrointestinal tracts (upper aerodigestive tract). The disease accounts for about 5% of all cancers and about 90% of all malignant tumors of the head and neck. In this category the most common tumors are of the larynx, the tongue, the floor of the oral cavity and palatine tonsil. These tumors are closely associated with well known causative factors such as tobacco smoking and alcohol abuse and develop most commonly in men in the sixth or seventh decade of life [53]. However, it is possible to define three groups of patients developing HNSCC infrequently—who are: people neither drinking nor smoking, women and young adults (YA). Young adults are patients aged younger than 45 (or 40) years (this age limit is not exactly fixed, but the majority of authors set the limit at 40 or 45 years). Such partition is used widely in medicine, not only in cancers of the head and neck but also in other neoplastic diseases (e.g., lung cancer) and in non-neoplastic diseases (e.g., heart diseases). In head and neck cancer patients this small group (about 5–10%) has attracted an attention of researchers a long time

ago [9, 34], but in spite of many studies (mainly clinical analyses) there is still no agreement concerning etiology and clinical features of HNSCC in YA. Some authors have noted worse results of treatment of HNSCC in YA than in older patients (OP) [5, 23, 28, 40, 47], which may be the effect of more aggressive character of the tumor [40] or more advanced stage of disease at the time of beginning of treatment (delayed contact with a doctor and late diagnosis) [23, 28]. However, many papers provided quite different findings including similar or even better outcome of treatment of HNSCC in YA than in OP [1, 4, 10, 19, 24, 32, 36, 42, 44, 50, 51] caused by better general health status implicating an opportunity for more aggressive treatment. A number of studies have also shown a lack of typical cancer causing factors (tobacco smoking and alcohol abuse) in YA [18, 19, 30, 31, 33, 42], that resulted in search for additional causative factors. The latter findings turned an attention for an increased genetic predisposition [2, 22, 27, 35, 39, 43, 48, 52], weakened immune systems [41, 45, 46] and narcotic abuse (marijuana and hashish) [3, 8]. Some authors, however, linked earlier incidence of HNSCC with a very high exposure to cigarettes and alcohol [26, 28].

To answer the question concerning the differences in the incidence and progression of HNSCC in YA we have undertaken the clinical analysis of 95 YA (≤45 years) and 95 matched OP with HNSCC, treated in Department of Otolaryngology and Laryngological Oncology of Karol Marcinkowski University of Medical Sciences in Poznan during the period 2000–2004 and have found some distinct differences between these groups [14, 15]. The conclusions from this clinical study were as followed. (1) In the etiology of HNSCC in YA the important role of typical exogenous factors (active and passive exposition to tobacco smoke and alcohol abuse) was observed. (2) Significantly higher alcohol consumption in YA than in OP may be one of the reasons of HNSCC in young age. (3) A frequency of familial cancer in YA with HNSCC seems to be higher than in OP, but further observations are needed. (4) In YA and particularly in patients younger than 40 years there was a higher percentage of patients with oral cavity cancer and lower percentage of patients with larynx cancer in comparison to OP group. (5) The tumors in YA were clinically more advanced than in OP, because YA delay the visit to physician, in spite of evident clinical symptoms. (6) HNSCC in YA were histological more mature and less malignant.

The study was aiming for the genetic factors associated with HNSCC in YA. Using various tests, the study compared the level of chromosome breaks (spontaneous and induced), the level of DNA damage (spontaneous and induced), DNA repair capacity and a distribution of polymorphic variants of glutathione transferases *M1* and *T1* genes.

Materials and methods

Patients

The study group consisted of 60 patients with HNSCC aged ≤45 years (YA) and the control group of 72 patients with HNSCC aged >45 years (OP), who were treated in the Department of Otolaryngology and Laryngological Oncology of Karol Marcinkowski University of Medical Sciences in Poznan during the period 2000–2004. All the patients were tobacco-smokers (mean number of cigarettes smoked daily in both groups was 22) and lived in the same region of Poland (Great Poland). The characteristic of analyzed groups is shown in Table 1. The study material was peripheral blood leukocytes.

Table 1 Characteristic of analyzed groups

Group of patients	Young adults			Older patients		
	Genotyping	Comet assay	Bleomycin test	Genotyping	Comet assay	Bleomycin test
Number of patients	60	30	20	72	30	20
Mean age (years)	41.5	40.8	40.8	62.9	61.3	60.8
Sex						
Male	51 (85.0%)	25 (83.3%)	17 (85.0%)	69 (95.8%)	29 (96.7%)	19 (95.0%)
Female	9 (15.0%)	5 (16.7%)	3 (15.0%)	3 (4.2%)	1 (3.3%)	1 (5.0%)
Localisation of tumor						
Oral cavity	10 (16.7%)	5 (16.7%)	4 (20.0%)	3 (4.2%)	2 (6.7%)	2 (10.0%)
Nasopharynx	2 (3.3%)	1 (3.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Oropharynx	3 (5.0%)	1 (3.3%)	1 (5.0%)	9 (12.5%)	6 (20.0%)	4 (20.0%)
Hypopharynx	1 (1.7%)	1 (3.3%)	1 (5.0%)	2 (2.8%)	0 (0.0%)	0 (0.0%)
Larynx	42 (70.0%)	21 (70.0%)	13 (65.0%)	58 (80.5%)	22 (73.3%)	14 (70.0%)
Other	2 (3.3%)	1 (3.3%)	1 (5.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

Bleomycin test was used to estimate chromosomal instability: spontaneous and induced by bleomycin in cells cultured in vitro [17, 20]. For every patient four cultures were maintained (20 cases of study group and 20 of control group, 72 h, 37°C). After 67 h to two of four cultures bleomycin (Bleocin—Nippon Kayaku Co., Ltd.; 30 mU/ml of culture) was applied to induce the aberrations and after 71 h to all cultures colchicine was added to stop the cell cycle at metaphase. Cultures were harvested using standard cytogenetic methods; the material was collected on slides and stained in a 5% solution of Giemsa's stain and analyzed by light microscope. For each patient 50 metaphases were estimated for spontaneous and 50 for induced chromosome breaks. Two parameters: b/c ratio (breaks per cell—a number of breaks of chromosomes in a cell) and the percentage of damaged cells were quantified. The scale of b/c values was described by Hsu and co-workers for induced aberrations as follows: b/c value <0.8 represents stable chromosomes, b/c ranging from 0.8 to 1.0 unstable chromosomes and b/c > 1.0 represents an increased chromosomal instability.

The comet assay allows estimating the spontaneous and induced DNA damage and DNA repair capacity [13]. Lymphocytes were isolated from circulatory blood (5 ml from 30 donors of study group and 30 of control group) and from these: (A) part was used to estimate spontaneous DNA damage, (B) part was exposed to the model mutagen bleomycin (induced damage) and (C) part was exposed to bleomycin and then incubated for 30 min at 37°C to estimate DNA repair. The lymphocytes were then embedded in agarose gel, lysed, denaturized, electrophoresed, neutralized, stained (DAPI) and analyzed by fluorescent microscopy. The length of 100 comets was determined for each sample.

Genotyping (60 patients of study group and 72 of control group). We analyzed the polymorphisms of glutathione transferases *M1* and *T1* genes involved in the detoxification process of metabolites of carcinogens of tobacco smoke [21]. DNA was isolated from circulatory blood by the phenol method and fragments to be tested were amplified by the PCR-multiplex technique. The presence (wild genotype) or the deletion (null genotype) of the analyzed genes was identified by agarose gel electrophoresis in the presence of control genes. We used the following primers: (A) for analysis of the polymorphism of *GSTM1* gene: J6, E7A and E7B for analyzed gene (132 bp) and BGI1 and BGI2 for control gene (β-globin gene—280 bp) and (B) for analysis of polymorphism of *GSTT1* gene: TLF and TLR for analyzed gene (480 bp) and VDRF and VDRR for control gene (800 bp).

Results

The results are shown in Tables 2, 3, 4, and 5.

(A) Bleomycin Test. The level of spontaneous and bleomycin-induced chromosome breaks (b/c ratio and percentage of damaged cells) was not significantly different in YA and OP groups (Table 2).

(B) Comet Assay. The levels of spontaneous and bleomycin-induced DNA damage, shown as an increase of migration of DNA fragments on electrophoresis gels, were very similar in both analyzed groups. DNA repair potential was also not significantly different in young and OP groups (Table 3).

(C) Genotyping. The distribution of polymorphic variants of *GSTM1* gene in YA and OP groups was significantly different ($P = 0.006$). Wild-type genotype representing efficient detoxification was present in 36.1% of OP and only in 15.0% of YA (Table 4). The distribution of polymorphic variants of *GSTT1* gene was similar in both analyzed groups (Table 5).

Conclusions

The number of chromosome breaks (spontaneous and induced), the level of DNA damage (spontaneous and induced), DNA repair potential and the distribution of polymorphic variants of *GSTT1* gene are not significantly different in YA and in OP, which suggests that these factors are

Table 2 Results of bleomycin test

	Group	*n*	Mean ± SD	Median	Statistical analysis
Spontaneous chromosome breaks—b/c ratio	YA	20	0.084 ± 0.063	0.080	ANOVA $P = 0.443$
	OP	20	0.070 ± 0.055	0.060	
Spontaneous chromosome breaks—% of damaged cells	YA	20	6.50 ± 4.10	5.00	Mann–Whitney test $P = 0.545$
	OP	20	5.55 ± 3.87	4.00	
Induced chromosome breaks—b/c ratio	YA	20	0.68 ± 0.61	0.50	ANOVA $P = 0.642$
	OP	20	0.77 ± 0.51	0.67	
Induced chromosome breaks—% of damaged cells	YA	20	34.25 ± 18.57	33.00	ANOVA $P = 0.978$
	OP	20	34.40 ± 15.15	36.00	

YA young adults, *OP* older patients

Table 3 Results of comet assay

	Group	*n*	Mean ± SD	Median	Statistical analysis
Spontaneous DNA damage (sample 0)	YA	30	60.0 ± 14.2	59.8	ANOVA $P = 0.734$
	OP	30	61.1 ± 10.9	59.9	
Induced DNA damage (sample BLM)	YA	30	73.4 ± 11.7	70.5	ANOVA $P = 0.675$
	OP	30	72.3 ± 7.9	72.1	
Reduction of DNA damage (DNA repair) (sample BLM—BLM-N)	YA	30	3.41 ± 3.79	3.17	ANOVA $P = 0.697$
	OP	30	2.97 ± 4.80	3.26	

YA young adults, *OP* older patients

Table 4 Results of genotyping—the distribution of polymorphic variants of *GSTM1* genes

Group	*GSTM1*		Total	Statistical analysis
	(−)	(+)		
YA	51 (85.0%)	9 (15.0%)	60 (100.0%)	χ^2-test $P = 0.006$
OP	46 (63.9%)	26 (36.1%)	72 (100.0%)	
Total	97 (73.5%)	35 (26.5%)	132 (100.0%)	

YA young adults, *OP* older patients

Table 5 Results of genotyping—the distribution of polymorphic variants of *GSTT1* genes

Group	*GSTT1*		Total	Statistical analysis
	(−)	(+)		
YA	12 (21.1%)	45 (78.9%)	57 (100.0%)	χ^2-test $P = 0.976$
OP	15 (20.8%)	57 (79.2%)	72 (100.0%)	
Total	27 (20.9%)	102 (79.1%)	129 (100.0%)	

YA young adults, *OP* older patients

not the causative agents of HNSCC incidence in young age. The important risk factor of HNSCC in YA may be *GSTM1* null genotype, which is associated with the deficit of detoxification of metabolites of polycyclic aromatic hydrocarbons present in tobacco smoke.

Discussion

The character of HNSCC in YA is a matter of some controversies. Typical and non-typical factors in etiopathogenesis and differences in clinical progression, as compared with those for OP, were discussed in many articles [1–5, 8, 10, 14, 15, 18, 19, 22–24, 26–28, 30–33, 35, 36, 38–48, 50–52]. It is not clear yet, if YA with HNSCC should be considered as a distinct group. A further question is and where to set the upper age limit (45, 40 or 35 years?). The authors who confirmed the worse results of treatment of HNSCC in YA have not agreed about the reasons of such situation. It may be the effect of more advanced stage of disease at the time of diagnosis or more aggressive character of the tumor connected with different etiological factors. In our own studies on YA with HNSCC we found an overfrequent familial incidence of head and neck cancer as compared with OP [14], which indicate for an important role of genetic background in YA. Genetic factors should be taken into account discussing both an incidence and progression of HNSCC in YA.

Until now a few papers concerning the role of genetic factor in the etiopathogenesis of HNSCC in YA have been published [2, 22, 27, 35, 39, 43, 48, 52]. Several studies have tested the effects of genetic alterations in genes involved in regulation of the cell cycle (*p53, p21, Rb, MDM2*) [2, 27, 39, 48], but the results of these studies were not convergent. Wang et al. [52] found that young patients with HNSCC more commonly than typical patients display microsatellite instability but there is no difference in distribution of polymorphic genes connected with DNA repair (*hMLH1 and hMSH2*). Jin et al. [22] looking a loss of heterozygosity in the neoplastic cells of the oral cavity and healthy epithelium demonstrated similar results in YA and typical patients.

Bleomycin test was used in patients with HNSCC by many investigators and most of them showed significantly more chromosome breaks in cancer patients than in healthy controls [7, 11, 49, 55]. In 1989 Schantz et al. [43] used the bleomycin test in the group of young people (age <40 years) with HNSCC and showed an increased level of induced chromosomal aberrations connected with a high degree of chromosomal instability (b/c ratio 0.96). The control group of young healthy subjects showed b/c ratio of 0.62 and young patients with non-squamous cell carcinoma of the head and neck showed b/c ratio of 0.50. In our studies we did not find chromosome instability to be a cause of HNSCC in YA. Contrary, a number of chromosomal breaks in YA group was lower than in OP. The difference can be explained first by the difference in control group. Schantz and co-workers compared YA with HNSCC with young healthy individuals and with young patients with non-squamous cell cancers of the head and neck, but we compared YA with OP with HNSCC. The results showed by Schantz do not answer the question about the differences in etiology of the HNSCC in YA and OP, because higher levels of chromosome instability in OP than in healthy controls were shown in many papers [7, 11, 49, 55]. Another reason of

discrepancy may be related to a small numbers of subjects included in the analyzed groups ($n = 20$) or a different age limit (Schantz—40 years, our group–45 years). However, in our group also the patients aged less than 40 years showed a number of chromosomal breaks lower than in the control group. Two another publications showed no differences in the results of bleomycin test depending on age [7, 49]. Cloos et al. [7] using bleomycin test indicated in a group of 313 patients with HNSCC and 334 healthy controls, that mutagen sensitivity is higher in patients than in controls, but does not depend on age in the both groups. In 2005 Székely et al. [49] published the results of bleomycin test used in patients with HNSCC in different localizations. They showed, that sensitivity to bleomycin in patients with oral and pharyngeal cancers is higher than in healthy controls (b/c 1.12–1.14 vs. 1.0) but on the other hand, sensitivity of laryngeal cancer patients is not significantly different when compared with healthy controls (b/c 1.05 vs. 1.0). The authors did not indicate any difference of b/c ratio depending on age and the ratio in a group of 39 YA (≤45 years) was not significantly different than in all groups of patients (b/c 1.08 vs. 1.11). Contrary to our results of bleomycin test are those concerning chromosomal aberrations induced by irradiation in patients with HNSCC published by Papworth et al. [35]. They showed significantly higher chromosome sensitivity to irradiation as well in G2 (chromosomal aberrations induction) as in G0 (micronuclei formation) phase in patients aged <45 years than in controls in the same age, but the comparison of OP and controls showed no differences in G2 phase and distinct smaller differences in G0 phase than in young patients and controls groups. In both these tests (G2 and G0 phase) the reverse correlation between age and radiosensitivity was shown in patients but not in controls. It is important to note, that these authors showed earlier an increased chromosome sensitivity to irradiation in peripheral blood lymphocytes (in G2 and G0 phase) of patients with breast cancer (the neoplasm, developed in tissue not directly exposed to environmental carcinogens), in which the results of bleomycin test were not increased when compared with healthy controls. As radiomimetic effects of bleomycin are well known, the reason of this differences is not clear and should be explained—may be irradiation causes some additional chromosomal aberration, and perhaps this technique fits better to study genetic predisposition of the neoplasms.

Comet assay is another technique used in the estimation of the role of genetic factor of HNSCC and other cancers. In HNSCC this technique was firstly used by Rao et al. [38] in patients with oral cancer. They showed significantly higher level of DNA damage in patients than in healthy controls and the results correlated with stage of clinical advance of the tumor. The following investigations were conducted in larynx cancer by Gajęcka et al. [13] and in lung cancer by Rajaee-Behbahani et al. [37]. Gajęcka et al. showed: (1) a higher level of spontaneous damage of DNA in patients than in healthy controls, (2) a higher level of DNA damage induced by bleomycin and benzo(a)pyrene in patients than in healthy controls and (3) less efficient repair of DNA damage caused by bleomycin and benzo(a)pyrene in patients than in healthy controls. Rajaee-Behbahani et al. showed: (1) similar level of spontaneous damage of DNA in patients and healthy controls, (2) higher in patients than in healthy controls level of bleomycin-induced DNA damage and (3) reduced repair of DNA damage caused by bleomycin in patients when compared with healthy controls. So far there were no investigation by comet assay technique in YA with HNSCC, but the results published by Gajęcka et al. [13] in patients with HNSCC and the results published by Rajaee-Behbahani et al. [37] in patients with lung cancer showed no correlation between age of patients and the level of spontaneous and induced damage of DNA and DNA repair potential. Our results also did not show any significant difference between YA and OP with HNSCC.

Genotyping—glutathione transferases *M1* and *T1* genes. The investigations of polymorphisms of glutathione transferases genes were preceded by analysis of phenotype of enzyme glutathione transferase M1. In 1993 Lafuente et al. showed a correlation between deletion phenotype of *GSTM1* and twofold increase of the risk of development of larynx cancer [25]. Two last metaanalyses showed a weak correlation between deletion genotypes of *GSTM1* and *GSTT1* and the risk of HNSCC, particularly high in the case of coexistence of deletion genotype of both genes [16, 54]. A significance of *GSTM1* and *GSTT1* genes in etiology of the HNSCC was studied extensively but we didn't find any study concerning their role in YA with HNSCC. However, Gajęcka et al. [12] compared the distribution of some genes responsible for carcinogens metabolism between patients with larynx cancer and healthy controls. They did not find any significant differences between all these groups but showed that the differences depend on age of analyzed patients. The polymorphism of *GSTM1* may be important in the group of young patients (≤49 years) (OR = 1.4) but not OP (≥50 years) (OR = 0.88), but the polymorphism of *GSTT1* may play a role in the group of OP (OR = 1.26) but not young patients (OR = 0.98). These results are in agreement with our results suggesting the important role of *GSTM1* but not *GSTT1* in etiology of HNSCC in YA. Moreover Canbay et al. [6] showed a significantly higher frequency of deletion genotype of *GSTM1* in the group of YA (<40 years) with thyroid cancer than in healthy controls. Also Liu et al. [29] showed in the group of patients with oral cancer that an overrepresentation of deletion genotype of *GSTT1* is connected with older age of patients.

In conclusion we think that genetic factor may play an important role in etiology of HNSCC in YA. Our results indicate that a significance of genetic factor not detectable at the general level (analysis of chromosome breaks, DNA damage and DNA repair capacity) was demonstrated at the specific (analysis of particular genes). However, the definition of the role of genetic factors in the etiopathogenesis of HNSCC in YA still requires further studies on larger groups of patients. In this context our work is getting a character of pilot study.

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