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SOMATIC MUTATION AND POLYMORPHISM ANALYSIS IN PLEOMORPHIC ADENOMAS OF THE SALIVARY GLANDS

SOMATSKE MUTACIJE I ANALIZA POLIMORFIZAMA U PLEOMORFNIM ADENOMIMA PLJUVAČNIH ŽLEZDA

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Summary

Background: Genetic studies of salivary gland neoplasms were mainly focused on chromosomal changes, and some specific patterns of chromosome translocations have been described. However, molecular alterations, in particular the role of HER-2/H-ras/c-myc signalling cascade in pleomorphic adenoma pathogenesis (PA), are less well characterized. In addition, data on single nucleotide polymorphisms (SNPs) as potential susceptibility factors for PA development are also quite scarce.
Methods: Mutational analyses were performed by means of real-time PCR (HER-2 and c-myc amplification analysis), PCR–SSCP and sequencing (H-ras point mutation detection). Polymorphisms analysis was performed by PCR–RFLP (survivin and MMP-9 genes).
Results: Amplification of HER-2 and c-myc has been found in 13% and 9% of PA cases respectively. Point mutations in H-ras codons 12/13 have been detected in 17% of PAs. No correlation could be established between these alterations and clinical characteristics of PAs, whereas they might play a role in a subset of malignant salivary gland tumours. As for survivin -31 G/C polymorphism, C allele carriers had a 4-fold decrease of the risk of developing PA ($p=0.05$). Carriers of the variant allele T of the -1562C/T SNP in MMP-9 gene had a 4-fold increase of the risk of developing PA ($p<0.001$).

Kratak sadržaj

Uvod: U ispitivanjima mehanizama nastanka tumora pljuvačnih žlezda uglavnom dominiraju citogenetičke studije, pa su tako detektovane i opisane različite hromozomske translokacije sa specifičnim obrascem javljanja. Međutim, molekularne promene u ovim tumorima i dalje su relativno slabo poznate, a pogotovo je malo podataka o potencijalnom značaju signalnog puta HER-2/H-ras/c-myc u razvoju i progresiji pleomorfnih adenoma (PA). Takođe, retki su i podaci o polimorfizmima pojedinačnog nukleotida (SNP) kao faktora predispozicije za nastanak PA.
Metode: Analize somatskih mutacija urađene su metodama *real-time* PCR (analiza amplifikacije HER-2 i c-myc), PCR–SSCP i sekvenciranja (detekcija tačkastih mutacija u H-ras). Analiza polimorfizama pojedinačnih nukleotida (SNP) vršena je primenom metode PCR–RFLP (u genima za survivin i MMP-9).
Rezultati: Amplifikacija gena HER-2 detektovana je u 13%, c-myc u 9% a tačkaste mutacije u kodonima 12/13 H-ras gena u 17% uzoraka. Nije ustanovljena veza između ovih promena i kliničkih odlika adenoma. Na malom uzorku karcinoma, pokazano je da je amplifikacija HER-2 povezana sa recidivima tumora. Nosioci C alela u -31G/C SNP gena za survivin imaju četiri puta manji rizik od nastanka PA ($p=0,05$), dok nosioci varijantnog alela T kod -1562 C/T SNP u MMP-9 genu imaju četiri puta veći rizik da obole od PA u odnosu na kontrolu ($p<0,001$).

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Conclusions: A longer follow-up of PA patients harbouring mutations could uncover a prognostic role of HER-2 and c-myc amplification as predictors of adenoma transformation into carcinoma. Both survivin and MMP-9 promoter polymorphisms represent susceptibility factors for the development of PAs in the Serbian population.

Keywords: pleomorphic adenoma, HER-2, c-myc, survivin, MMP-9

Zaključak: Dužim praćenjem pacijenata sa PA mogla bi da se ustanovi prognostička uloga HER-2 i c-myc amplifikacija kao indikatora za transformaciju adenoma u karcinom. Polimorfizmi u promotorima gena za survivin i MMP-9 predstavljaju modulatore rizika za razvoj pleomorfnih adenoma u srpskoj populaciji.

Ključne reči: pleomorfni adenom, HER-2, c-myc, survivin, MMP-9

Introduction

Salivary gland (SG) tumours are a highly heterogeneous group of tumours and include more than 35 histological subtypes, from benign adenomas to high-grade carcinomas.

Pleomorphic adenoma (PA) is the most common salivary gland tumour representing about a half of all salivary gland neoplasms and 65% of parotid gland tumours (1–3). Pleomorphic adenoma (PA) is a benign mixed salivary gland tumour, associated with abnormal karyotypes in up to 70% of cases, with non-random involvement of 8q12, the locus of the pleomorphic adenoma (PLAG1) gene.

The vast majority of pleomorphic adenomas occur in the parotid glands, but they can also be found in the submandibular glands, sublingual glands, or small salivary glands. They mostly arise between the ages of 30 and 60 years and are more commonly found in females than in males (4). Histologically, they are characterized by variable patterns formed by both epithelial and myoepithelial cells in a mucoid/myxoid, chondroid, or hyalinised stroma. The main therapy is surgical removal of the tumour with surrounding salivary gland tissue. Some 2 to 17% of all pleomorphic adenomas tend to the undergo a malignant transformation, giving rise to the so-called »carcinoma ex pleomorphic adenoma« (CXPA) (3–5).

Previous studies of the pathogenesis of pleomorphic adenomas were mainly focused on chromosomal changes and have shown that PA are characterized by highly specific patterns of chromosome translocations, preferentially affecting the DNA-binding transcription factor genes PLAG1 and HMGA2 (6–8). Molecular changes in PA, however, are not well characterized and, also, very little is known about the genetic events leading to their transformation into carcinomas (9–13).

Activation of oncogenes, when coupled with inactivation of tumour suppressor genes, leads to uncontrolled cell proliferation. One of the commonly activated signalling pathways in tumorigenesis is the HER-2/H-ras/c-myc pathway.

Human epidermal growth factor receptor-2 (HER-2, also known as c-erbB-2 or neu) gene is a proto-oncogene located on chromosome 17 and it encodes a 185-kd transmembrane tyrosine kinase receptor (14). HER-2 is a member of the epidermal growth factor receptor family and is recognized as a key oncogene in several malignancies (15). Amplification of the HER-2 gene and overexpression of the HER-2 protein have been observed in various solid tumours (16–18).

H-ras oncogene, located on chromosome 11, is functionally related to HER-2. The protein product of H-ras oncogene – p21, transmits signals via Raf/MAPK signalling cascades to various transcription factors (19). H-ras is usually activated by point mutations in codons 12, 13 and 61.

The c-myc oncogene encodes a transcription factor with an essential role in cell proliferation, cell growth, differentiation and apoptosis (20, 21). Its protein product controls the expression of 10–15% of all mammalian genes, weather as an activator or as a repressor (22). The most common c-myc aberration leading to its activation in solid tumours is gene amplification (23). It is also functionally related to HER-2 and H-ras. To date, little is known about the involvement of HER-2/H-ras/c-myc signalling cascade in the development of PAs and about its possible prognostic significance in both PAs and CXPAs (10, 24, 25).

PA, like the vast majority of tumours, is a multifactorial, polygenic disease, and heredity may represent an important factor in its development. Although gene polymorphisms are an expression of normal variations in the hereditary basis, their effect on the phenotype is interesting, especially the association with susceptibility to certain diseases (26). Specifically, predisposition to PA could be modulated, among others, by functional polymorphisms in the genes related to mechanisms, more or less intrinsic to salivary gland tumorigenesis.

Survivin, a key regulator of mitosis and programmed cell death, has been shown to play a prominent role in the promotion of tumorigenesis. Changes in its expression may be the consequence of gene amplification, hypomethylation, etc. In some instances, they may be due to a common -31 G/C single nucleotide polymorphism (SNP) at the CDE/CHR repressor binding motif of the survivin gene promoter (27). Consequently, functional polymorphisms influencing survivin expression may thus be considered as risk factors for carcinogenesis (28).

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases subdivided into 5 major groups, capable of degrading almost all components of the extracellular matrix including interstitial and basement membrane collagens, fibronectin, laminin and proteoglycan core protein. They are involved in connective tissue remodelling and degradation. MMP 9 contributes to carcinogenesis, tumour growth, invasion and angiogenesis. A SNP in the promoter region of the MMP 9 gene (-1562 C/T) may influence tumour occurrence and progression via modifying mRNA transcription and protein expression. The C-T base substitution leads to increased transcriptional activity and genotypes with a T allele (CT, TT) have higher enzymatic activity (29).

The aims of this study were to: 1) determine the importance of mutations in HER-2, H-ras and c-myc genes in pleomorphic adenoma pathogenesis; 2) establish the role of SNPs in the survivin and MMP 9 genes as putative susceptibility factors for pleomorphic adenoma development in the Serbian population.

Material and Methods

Samples

In total, 54 PA and 6 CXPA samples from patients treated at the Clinic of Maxillofacial Surgery, School of Dental Medicine, University of Belgrade (2007–2010), were included in this study. The study was performed in compliance with the Declaration of Helsinki ethical guidelines and approved by the Ethics Committee of the home institution. All participants have signed an informed consent form. The DNA was extracted from formalin-fixed, paraffin-embedded samples. Deparaffinization was carried out by two immersions in xylene followed by rehydration in absolute and 70% ethanol. Total genomic DNA was obtained using a standard phenol/chloroform extraction protocol. For the association study, a larger number of both PA and control DNA samples was used (74 PAs and 127 controls for survivin SNP analysis and 51 PAs and 101 controls for MMP-9 SNP analysis).

Real–time PCR

A real–time polymerase chain reaction (qPCR) – comparative Ct method of quantitation (ΔΔCt) was performed using Maxima^{TM} SYBR Green qPCR Master Mix(2X) (MBI, Fermentas, Vilnius, Lithuania). Primer sequences were HER-2: F 5'CCTCTGACGTCCATCATCT3', R 5'ATCTTCTGCTGCCGTCGTT3'; c-myc: F 5'GCTCCAAGACGTTGTGTGTTCG3', R 5'GGAAGGACTATCCTGCTGCCAA3'. A single-copy gene encoding dopamine D2 receptor (D2R) was used as a reference gene in this study. Primer sequences for the D2R gene were: F 5' CCACTGAATCTGTCCTGGTATG 3', R 5' GTGTGGCATAGTAGTTGTAGTGG 3'. qPCR reactions for all genes for each sample were carried out in duplicate, using 20 ng DNA as a template. To confirm the specificity of the amplified product, we performed the melting curve analysis in each case. The ΔΔCt was calculated separately for HER-2, c-myc and the D2R gene and the amplification levels of each sample were normalized against D2R as a reference gene. A gene dose greater than 2.5 was considered amplified.

Polymerase chain reaction–single strand conformation polymorphism (PCR–SSCP)

The screening for the presence of the most common mutations in codons 12 and 13 of the H-ras oncogene was done using PCR amplification followed by SSCP analysis. PCR reaction was performed in a volume of 25 μL reaction mixture containing 300 ng of genomic DNA and 200 nM of the following primer pair: F 5'ATGACGGAATATAAGCTGGT 3' and R 5' CGCCAGGCTCACCTCTATA 3'.

PCR conditions were: initial denaturation step at 95 °C (3 min), followed by 35 cycles at 95 °C (30 s), 50 °C (30 s), 72 °C (30 s) and final extension at 72 °C (7 min). The amplified product of 123 bp was visualized using 8% polyacrylamide gel electrophoresis and ethidium bromide staining. For further SSCP analysis, 5 μL of PCR product were mixed with 10 μL of loading dye (95% formamide, 20 mmol/L EDTA, 0.05 xylene cyanol, 0.05% bromophenol blue). The samples were denaturated in the thermal cycler by heating to 96°C for 8 min, then loaded onto 10% non-denaturating polyacrylamide gel. Gels were stained with 2% $AgNO_3$. The presence of mobility shift of bands was an indication of mutation. A PCR product of a sample obtained from the blood of a healthy subject was used as a negative control.

To maximize the accuracy, each sample was tested at least twice by separated PCR reactions and SSCP runs. In order to confirm the results of PCR–SSCP, DNA samples were sequenced commercially.

Survivin-31 G/C (rs9904341) genotyping

Survivin promoter polymorphism at position -31 was determined by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP). Primers F 5'-AAGAGGGCGTGCGCTCCCGACA-3' and R 5'-GAGATGCGGTGGTCCTTGAGAAA-3' generated a fragment surrounding -31 G/C SNP of 151 bp. PCR was performed in a total volume of 20 μL containing 2 μL of 10 × PCR buffer (MBI, Fermentas, Vilnius, Lithuania), 1.5 mmol/L of $MgCl_2$, 0.2 mmol/L dNTPs, 0.375 μmol/L of each primer, 200 ng of genomic DNA and 1 unit of Taq DNA polymerase (MBI, Fermentas, Vilnius, Lithuania). The amplification conditions for -31 G/C were as follows: initial denaturation at 95 for 5 minutes, followed by 35 cycles consisting of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, elongation at 72 °C for 1 min, and a final elongation at 72 °C for 10 min.

The amplified fragment was digested with 5 units of Msp I (MBI, Fermentas, Vilnius, Lithuania), resulting in products of 151 base pairs (bp) for the GG genotype, two fragments of 90 and 61 bp for the CC genotype and three fragments of 151, 90 and 61 bp for the CG genotype.

MMP-9 -1562 C/T (rs3918242) genotyping

The sequence surrounding the SNP position in the MMP-9 gene promoter was amplified using the primer pair: F 5′-GCCTGGCACATAGTAGGCCC-3′ and R 5′-CTTCCTAGCCAGCCGGCATC -3′. PCR was carried out in a total volume of 25 μL, containing 300 ng genomic DNA, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 μmol/L of each primer, 200 μmol/L each dATP, dCTP, dGTP and dTTP, and 2.5 U Taq DNA polymerase (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The solution was incubated for 3 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 45 s at 65 °C and 45 s at 72 °C, with a final extension of 72 °C for 7 min.

Each PCR product was digested with three units of SphI (MBI, Fermentas, Vilnius, Lithuania) overnight and the fragments were separated on an 8% polyacrylamide gel stained with ethidium bromide. After digestion, wild type homozygotes (CC) showed 1 band of 435 bp, mutated homozygotes (TT) had 2 bands (247 and 188 bp) and heterozygotes (CT) had 3 bands (435, 247 and 188 bp).

Genotypes were confirmed by randomly re-genotyping 10% of the samples. There were no discrepancies between the genotypes determined in duplicate.

Statistical analysis

Chi square test was used to determine possible differences in the genotype and allele frequencies. The association of -31 survivin and MMP-9 variants with risk of disease was examined by use of unconditional logistic regression analysis to calculate odds ratios (OR) and their 95% confidence intervals (CI). P values of <0.05 were considered statistically significant.

The expected frequency of survivin and MMP-9 variants in controls was analyzed by the Hardy-Weinberg equilibrium test. Calculations were performed with the statistical package Stata V6.

Results

Mutational analysis

Amplification of HER-2 was identified in 7 out of 54 (13%) cases of PA and in 2 out of 6 (33%) cases of CXPA. MYC was amplified in 5 out of 54 (9%) cases of PA and in 2 out of 6 (33%) cases of CXPA.

H-ras codon 12/13 mutations were found in 9 cases of PA (16.7%) and none of CXPA. Mutational analysis and epidemiological data are summarized in *Table I*. There was no statistically significant difference between molecular findings in different clinical subgroups. SSCP analysis also detected an H-ras codon 27 (His27His) polymorphism in exon, in 19 out of 54 cases (35%). This finding was confirmed by RFLP (data not shown).

Table I Molecular findings in relation to clinical and epidemiologic data in 54 cases of salivary gland pleomorphic adenoma.

Variable	Patients No. (%)	HER-2 Amplification	MYC Amplification	H-ras mutation
Sex				
Male	10 (19)	1/10	0/10	2/10
Female	44 (81)	6/44	5/44	7/44
Age				
<40	24 (44)	4/24	3/24	3/24
40–60	23 (43)	1/32	1/23	4/23
>60	7 (13)	2/7	1/7	2/7
Size, cm				
<2.5	12 (22)	1/12	2/12	2/12
2.5–3.5	36 (67)	6/36	3/36	5/36
>3.5	6 (11)	0/6	0/6	2/6
Duration of symptoms, months				
<12	19 (35)	4/19	1/19	4/19
12–36	18 (33)	1/18	1/18	1/18
>36	17 (32)	2/17	3/17	4/17
Smoking				
Yes	24 (44)	2/24	3/24	4/24
No	30 (56)	5/30	2/30	5/30

Table II Genotype and allele frequencies and logistic regression analysis data for –31 G/C survivin gene and –1562 C/T MMP-9 gene polymorphisms.

Survivin –31 G/C genotype	PA (n=74)	Control (n=127)	OR	95% CI	p
GG	32 (43%)	51 (40%)	Reference		
GC	40 (54%)	63 (50%)	1.01	0.56–1.83	0.54
CC	2 (3%)	13 (10%)	0.25	0.05–1.16	0.050*
GC + CC	42 (57%)	76 (60%)	0.88	0.49–1.57	0.388
allele					
G	0.70	0.64	Reference		
C	0.30	0.36	0.76	0.42–1.38	0.23
MMP9 –1562 C/T genotype	PA (n=51)	Control (n=101)	OR	95% CI	p
CC	27 (53%)	83 (82%)	Reference		
CT	22 (43%)	17 (17%)	3.98	1.85–8.57	<0.001*
TT	2 (4%)	1 (1%)	6.1	0.54–70.50	0.16
CT + TT	24 (47%)	18 (18%)	4.1	1.94–8.67	<0.001*
allele					
C	76 (75%)	183 (91%)	Reference		
T	26 (25%)	19 (9%)	3.37	1.48–7.66	0.002*

PA – pleomorphic adenoma, OR – odds ratio, CI – confidence interval

Polymorphisms analysis

The observed genotype frequencies in the case and control groups were in the Hardy-Weinberg equilibrium. The genotype and allele frequency distribution and risk estimates of survivin -31 G/C polymorphism are given in *Table II*. In terms of susceptibility, individuals homozygous for C have approximately a 4-fold decrease in the risk for developing PA, compared to GG homozygotes (p=0.05). The C allele obviously exhibits a protective effect in its carriers.

A significant difference in genotype and allele frequencies was also found between the PA group and controls for the -1562C>T SNP (p<0.001). Carriers of the variant allele T had roughly a 4-fold increase in susceptibility for PA compared to wild type homozygotes (CC). The observed genotype and allele frequency distribution and risk estimates are given in *Table II*.

Discussion

Though alterations of oncogenes and tumour suppressor genes have been implicated in the development of salivary gland tumours, very little is still known about the possible role of the HER-2/H-ras/c-myc signalling cascade in PAs.

The present study showed that the magnitude of molecular changes in the HER-2/H-ras/c-myc pathway was rather modest in PAs and somewhat higher in carcinomas, though the latter results are not fully reliable due to the small sample size. Amplification of HER-2 was identified in 13% cases of PA and in 33% of CXPA cases. C-myc was amplified in 9% of PAs and also in 33% of CXPA cases. The gain of genes or chromosome regions commonly occurs in tumour cells, but a recent study using FISH did not show evidence of HER-2 gene amplification in benign pleomorphic adenomas (30). Nonetheless, HER-2 amplification has been previously indicated as a common event in the process of PA transformation into carcinoma ex pleomorphic adenoma (10). It has also been shown that HER-2 and TP53 are synergistically involved in the early stages of malignant transformation of PA (25, 31). C-myc amplification has previously been reported in some cases of CXPA, but never in benign PA (32, 33). In the present study, HER-2 and c-myc amplification were a rare event, but one cannot exclude a subgroup of PA in which this signalling cascade is activated. It must be emphasized that HER-2 amplification was often related to recurrences in malignant tumours. One carcinoma with both genes amplified was metastatic, though the preceding benign tumour revealed no amplification of HER-2 nor c-myc oncogenes. Some researchers suggest that HER-2 plays a role in the progression of carcinoma ex PA, and that the presence of HER-2 amplification might be an indicator of poor prognosis, which is corroborated by the present study (34).

Our results on H-ras mutations incidence in PA, though lower (17%) than the results previously reported for the same population (35%) by Milasin et al. (35), are not negligible and point to a possible invol-

vement of this specific molecular change in the pathogenesis of a particular PA subgroup. Conversely, Augello et al, (11) who detected a mutated H-ras gene in only 4% of PAs, concluded that Ras mutations are irrelevant in PA pathogenesis. Though none of the gene alterations could be related to the clinical characteristics of PAs, a potential implication of these mutations in malignant transformation of adenomas into carcinomas cannot be excluded, making essential an extended follow-up of the patients harbouring mutations.

Contrasting with oncogene analysis, polymorphisms analysis gave more satisfactory results. Only a few association studies trying to correlate gene polymorphisms and risk of PA have been carried out and none concerning survivin and MMP-9 gene promoter SNPs.

It was suggested that survivin, a member of IAPs (Inhibitor of Apoptosis Protein) family which functions both as a promoter of cell proliferation and inhibitor of apoptosis is overexpressed in various malignancies, but also in some benign tumours (36, 37). Our study indicates that carriers of the C allele have a 4-fold decrease in PA susceptibility compared to GG homozygotes ($p=0.05$), i.e. that the C allele has a protective role. Similar results have already been obtained for another type of benign tumours in the Serbian population, the keratocystic odontogenic tumours (38), as well as in one type of malignancy – Wilm's tumours (39).

The SNP –1562 C/T in the promoter of the MMP-9 gene was shown to upregulate gene transcription, which in turn leads to increased biosynthesis of the enzyme with high potential to degrade the connective tissue matrix. In the study of Zhang et al. (40) higher levels of MMP-9 expression have been observed in PA, compared to normal salivary gland tissue, which is in line with our results. Namely, we observed a 4-fold increase of PA risk in heterozygous carriers of the variant T allele, which is responsible for the transcription upregulation, indicating a strong association of the -1562 single nucleotide polymorphism in the MMP-9 gene promoter with the development of PA in the Serbian population.

Despite numerous studies, the natural history of pleomorphic adenomas, the most common salivary gland tumors, remains unclear. It seems, however, that HER-2 amplification emerged as a valid marker of salivary gland tumour aggressiveness. In addition, detection of specific polymorphisms might help in the identification of patients particularly inclined to develop pleomorphic adenomas.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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