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Evaluation of CDH1 Promoter Methylation and HPV Infection Status in the Development of Parotid Pleomorphic Adenoma

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ABSTRACT Both benign and malignant salivary gland tumors are clinically heterogeneous and they display different histology. Researchers aimed to elucidate the potential roles of both CDH1 methylation and high-risk oncogenic human papilloma virus (HPV) types HPV16 and HPV18 in parotid pleomorphic adenoma. Genomic DNA samples extracted from formalin-fixed paraffin-embedded (FFPE) samples of parotid pleomorphic adenoma and control tissues were subjected to methylation specific PCR (MSP) for hypermethylation analyses. HPV infection presence was evaluated using both GP5+/GP6+ consensus primers and type-specific primers for HPV16 and HPV18. Both CDH1 promoter hypermethylation and HPV16 infection partially seemed to be responsible in the occurrence of the tumors. According to the best of the researchers' knowledge, this is the first study investigating the possible roles of both CDH1 methylation and HPV involvement in parotid pleomorphic adenoma. Multicentered studies with larger sample cohorts could be highly beneficial to strengthen this study's results and thus draw a more precise conclusion

INTRODUCTION

Benign and malignant salivary gland tumors are rare forms of head and neck tumors and benign cases constitute the greatest frequency since only 20 percent are malignant. An overall European standardized rate of 4.2–4.9 per 100,000 person-years was reported with a female preponderance (1:1.43) and with an annual 1 percent rise in female incidence (Valstar et al. 2017). In a detailed review study of 12-years period conducted in Turkey, the prevalence of salivary gland neoplasm was reported as 0.09 percent (235/244.204 cases) with a female-to-male ratio of 1.04:1 and of the 235 neoplasms, 159 samples (67.66%) were reported to be located in the parotid gland (Etit et al. 2012).

Salivary gland neoplasms can sometimes represent a diagnostic and therapeutic challenge because of histological heterogeneity and thus a better understanding of molecular mechanisms may offer improved treatment outcomes (Nikolic et al. 2015).

CDH1 as a tumor suppressor gene plays a role in cell-to-cell adhesion and is important in intercellular junction complex formation as well as the adhesion of epithelial cells and is also believed to play an important role in critical stages of lip and palatal formation. Either mutations or changes in DNA methylation may have the potential to alter gene expression of CDH1 (Tania et al. 2018).

Papillomaviruses are members of the *Papovaviridae* family and HPV is a relatively small, nonenveloped virus, 55 nm in diameter. The HPV genome consists of a single molecule of double-stranded, circular DNA containing approximately 7.900 bp associated with histones (Burd et al. 2003). Human papillomaviruses are the causative agents of a variety of human cancers, with cervical cancer being the most prevalent. All papillomaviruses encode four conserved core pro-

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teins: E1 and E2 replication factors and L1 and L2 capsid proteins. E4, E5, E6 and E7 accessory proteins are encoded by the oncogenic HPVs (McBride 2017). Tumor development requires the combined action of two viral oncoproteins, E6 and E7 which manage a number of critical cellular control pathways, and thus ultimately result in the development of malignancy (Thatte and Banks 2017).

Objectives

The aim of this study was to determine some specific molecular parameters; respective roles of CDH1 promoter methylation status and HPV prevalence in the development of parotid pleomorphic adenoma. In the light of literature data reflecting the possible roles of CDH1 and HPV infection in a variety of tumors, it sounds reasonable to investigate the roles of both in parotid adenoma.

Since the molecular studies with parotid pleomorphic adenomas are very rare, the researchers focused on this field to discover these possible parameters in the progression of this neoplasm.

MATERIAL AND METHODS

Histopathological and Clinical Data

A total of 23 tumor cases with pleomorphic adenoma of the parotid gland and as control group, 10 normal parotid gland tissues of the individuals who underwent biopsy with suspect of pleomorphic adenoma were recruited. Tumor specimes were obtained by a retrospective compilation from the records of the pathology department of the Goztepe Research and Training Hospital, Istanbul Medeniyet University (Istanbul, Turkey). The study was approved by the Institutional Ethical Committee of the university (Approval number: 2019/0300).

DNA Extraction From Tissues

Tissue samples collected from the participants were subjected to DNA isolation with Magnesia® 16 Nucleic Acid Extraction Instrument with the compatible kit to the instrument. DNA concentrations and purities were measured by a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.).

Analysis of CDH1 Promoter Methylation Status

The promoter metyhlation status of CDH1 (E-cadherin) was determined by methylation specific PCR. Genomic DNA extracted from FFPE samples was modified by sodium bisulfite treatment with EZ-DNA Methylation-Gold™ Kit (Zymo research, Orange, CA, USA) according to the manufacturer's instructions. PCR reactions were performed separately to avoid misinterpretation with primers designed for methylated and unmethylated promoter region previously described (Liu et al. 2016: CDH1-MF: 5'-TTAGGTTAGAGGGTTATCGCGT-3' and CDH1-MR: 5' -TAACTAAAAATTCACCTACCGAC-3' which represent methylated primers and CDH1-UF: 5'-TAATTTTAGGTTAGAGGGTTATTGT-3' and CDH1-UR: 5' -CACAACCAATCAACAACACA-3' which are used for unmethylated condition). The sodium bisulfite-converted DNAs were amplified with hot-start ZymoTaq™ DNA Polymerase (Zymo Research, Orange, CA, USA). PCR reaction mix (50 µl) included 1x reaction buffer, 0.25 mM dNTPs, 1.0 µM primers, 2U Zymo Taq polymerase and 3 µl of converted DNA templates. The cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C denaturation for 30 sec, annealing at 60°C (both for unmethylated and methylated sequences) for 40 sec, 72°C extensions for 1 min, and final extension at 72°C for 7 min. The post MSP products were loaded on a 3 percent molecular biology grade agarose gel, stained with ethidium bromide, electrophoresed and analyzed with gel documentation system (SYNGENE Ingenius 3, England). Universal methylated human DNA standard (250 ng/µl) (Zymo research, Orange, CA, USA) was used as a positive control for methylation, and water was used as a negative control for PCR. The 50-bp DNA ladder was used as a marker. Methylation status of the gene was indicated as methylated when amplification products were detected in reactions with the primers M or both M and U. Unmethylation status was defined when amplification products were detected in reaction with the primers U only. Re-amplification of all the

samples was done to confirm the findings and there was no discrepancy in duplicates.

Identification of HPV Types

All samples were searched for HPV infection by PCR with the GP5+/GP6+ consensus and type-specific primers for HPV16 and HPV18. Primer sequences used in the study were previously described (Shikova et al. 2009; consensus primers GP5+ and GP6+ sequences were 5'-TTTGTTACTGTGGTAGATACTAC-3', 5'-GAAAAATAAACTGTAAATCATATTC-3', respectively. HPV primers: HPV16-F: 5'-TGCTAGTGCTTATGCAGCAA-3', HPV16-R: 5'-ATTTACTGCAACATTGGTAC-3'; HPV18-F: 5'-AAGGATGCTGCACCGGCTGA-3', HPV18-R: 5'-CACGCACACGCTTGGCAGGT-3'). A total of 25 µl PCR reaction mix included 1x buffer, 20 mM $MgCl_2$, 0.5 µl dNTP, 10 pmol primers, 25 ng T432 protein, 1.5 U Taq polymerase (Thermo Fisher Scientific, Inc.) and 100 ng DNA. PCR conditions were previously described (Freitas et al. 2007). Briefly, PCR condition for GP5+/GP6+ consensus primers were as follows: 40 cycles of denaturation for 1.0 min at 94°C, annealing for 1.0 min at 45°C and extension for 1.0 min at 72°C. Additional PCR reactions were also conducted to investigate the presence of oncogenic HPV types, HPV16 and HPV18 with similar conditions to those used for GP5+/GP6+ consensus primers, except with the difference in annealing temperatures (HPV16: 57°C, HPV18: 65°C). PCR products were loaded on 2 percent molecular biology grade agarose gel stained with ethidium bromide, electrophoresed and analyzed under the UV light. To control the success of repeat analysis, all samples were genotyped again and the results yielded 100 percent concordance.

Statistical Analysis

Variables were evaluated by Pearson linear correlation by PAST Paleontological Statistics Version 3.18 program and graph format of Pearson matrix was made with the same program. All statistical analyses were done using Statistical Package for Social Science (SPSS software package, Version 21.0; SPSS Inc., Chicago, IL, USA). The differences of gene methylation status between variables were assessed using Pearson's $\chi 2$ test unless the smallest expected value was < 5, in which case Fisher's exact test was used. P <0.05 was considered statistically significant.

RESULTS

Analysis of CDH1 Promoter Methylation Status

CDH1 promoter methylation increased in parotid gland neoplasms since 26.08 percent (6/23) of the samples were methylation positive while hypermethylation was observed in 10 percent (1/10) of the control samples. CDH1 methylated and unmethylated products were identified by the amplicon lengths of 116 bp and 97 bp, respectively (Fig. 1). Methylation status

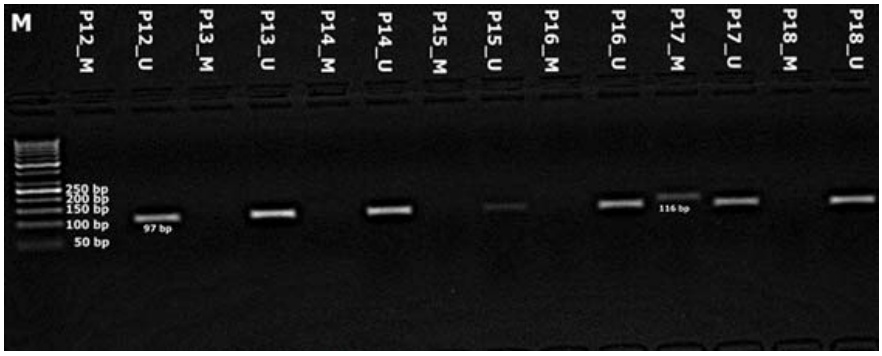

Fig.1. Representative example of methylation-specific polymerase chain reaction for gene CDH1 (alias E-CAD) (Gel image by the authors Ozen et al.)
Lanes M and U correspond to methylated and unmethylated reactions, respectively. In the picture, only one sample (P17) is methylated out of 7 samples. On the left: molecular weight marker; on the right: the size of each PCR product, methylated and unmethylated

strongly showed negative correlation with drug use (a general use of antidiabetic, antihypertensive, and thyroid drugs) and smoking status (Fig. 2) while a correlation was not present between hypermethylation profile and other factors such as HPV infection status.

HPV Infection Status

All samples were searched for HPV infection with the GP5+/GP6+ consensus primers amplifying a 150 bp fragment of the L1 HPV genomic region and type-specific primers for high-risk HPV16 and HPV18, amplifying DNA fragments of 152 bp and 216 bp, respectively (Fig. 3). HPV infection was detected in 13.04 percent (3/23) of the tumor samples and 30 percent of the control samples (3/10) by PCR technique with GP5+/GP6+ consensus primers. Regardless of the situation of the PCR results with GP5+/GP6+ consensus primers, all samples were also subjected to PCR analysis with type-specific primers for oncogenic HPV16 and HPV18 detection. 21.73 percent (5/23) of the parotid pleomorphic adenoma samples were positive for HPV16 infection while the ratio was 0 percent (0/23) for control samples reflecting HPV16 infection as an increased risk factor for the development of parotid pleomorphic adenoma. GP5+/GP6+ mediat-

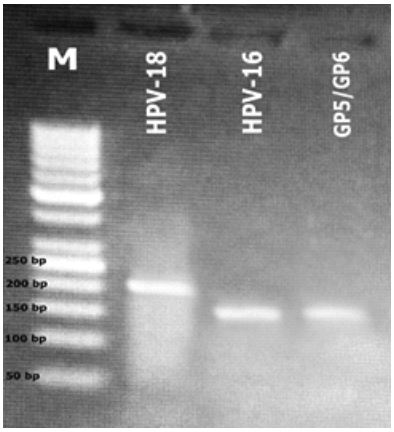

Fig. 3. HPV detection by consensus primers and type-specific primers for HPV18 and HPV16 detection
M: Molecular weight marker; HPV-18: Amplicon reflecting the size of 216 bp; HPV-16: Amplicon reflecting the size of 152 bp; GP5/GP6: PCR with GP5+/GP6+ primers yielded an amplicon of 150 bp (Gel image by the authors Ozen et al.)

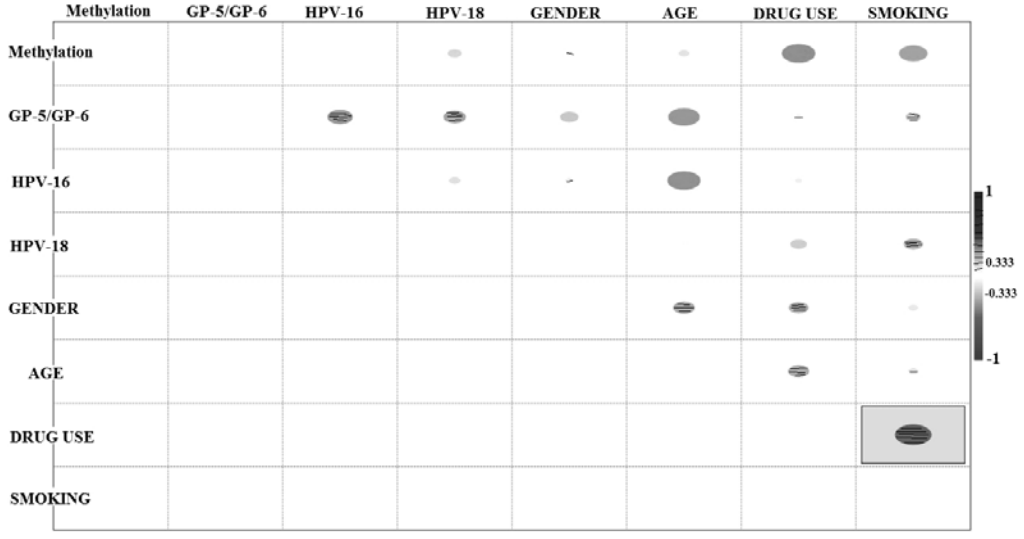

Fig. 2. Pearson correlation coefficient graph between variables (Values in the box represent p<0.05)
Methylation status is strongly negatively correlated with drug use (a general use of antidiabetic, antihypertensive, and thyroid drugs) and smoking status. There is no correlation between hypermethylation profile and other factors such as HPV infection status (Striped circles show the positive correlation).

ed PCR could not detect 3 positive HPV16 cases reflecting the need of the combined use of different consensus primers explained in discussion part. HPV18 infection ratios were 4.34 percent (1/23) for tumor samples and 10 percent (1/10) for control samples. The only HVP18 positive tumor sample was the one that was not detected with GP5+/GP6+ mediated PCR while HPV18 positive control sample was also positive with GP5+/GP6+ consensus primer pair. It seems that HPV18 infection does not play an important role in the development of parotid pleomorphic adenoma development in contrast to HPV16 infection.

DISCUSSION

Carcinogenesis is a multistep process which includes the accumulation of both genetic and epigenetic alterations. Recent cancer studies not only aim to investigate mutations and/or expression changes on mRNA and/or protein levels, but also aim to draw a more comprehensive picture via targeting epigenetic alterations which consist of gains and losses of DNA methylation and histone modifications. Thus, one of the aims of the researchers was the investigation of the CDH1 hypermethylation profile since very few methylation studies exist in literature related with salivary gland tumors. Moreover, DNA methylation is known to be involved in tumor development and progression and evaluation of the methylation patterns of the tumor suppressor genes may be an ideal tool for early diagnosis of various cancer types. In the study of Nikolic et al. (2015), the inactivation of tumor suppressor genes p16 and p14 by promoter hypermethylation was shown to be as an important mechanism in the development of both pleomorphic adenomas and carcinoma ex pleomorphic adenomas in Serbian population. Very high RUNX3 methylation percent was reported in salivary gland adenoid cystic carcinoma samples when compared with the corresponding normal salivary glands and also methylation status was a significant predictor of 5-year disease-free survival following surgery (Ge et al. 2011). In salivary duct carcinoma of the parotid gland, methylation of galanin receptors was associated with a significant decrease in overall survival (Kanazawa et al. 2018). Hypermethylation of p14 was an important process in the development of salivary gland mucoepidermoid carcinoma (Nikolic et al. 2018). Besides with these methylation studies investigating some important suppressor genes in salivary gland diseases, relatively very few methylation studies exist on CDH1 (E-cadherin) which was researchers' target in this study. CDH1 gene is located on chromosomal region 16q22.1 and encodes a 120 kDa transmembrane glycoprotein. As a tumor suppressor gene CDH1 plays a major role in invasion and metastasis of various human cancers (Shen et al. 2016). CDH1 promoter methylation was shown to play an important role in tumor cell differentiation and perineural invasion of human salivary gland adenoid cystic carcinoma (Zhang et al. 2007). CDH1 methylation frequency was implicated to be significantly high in oral cavity tumors compared with the paired surgical margins (Strzelczyk et al. 2018). CDH1 hypermethylation rate was found as high as 88.3 percent in head and neck cancer (Calmon et al. 2007). Though the studies in literature predominantly indicate the increase of methylation rate in head-neck tumors, some exceptions also exist as in the case of the study of Marsit et al. (2008) who proposed CDH1 hypermethylation as an independent predictor of improved survival in head and neck squamous cell carcinoma and as in the case of the study of De Schutter et al. (2009) who predicted epigenetic silencing of CDH1 with a better outcome in patients with advanced head and neck squamous cell carcinoma treated by radiotherapy only. Though some exceptions related with head and neck carcinomas also exist, researchers' results seem to be in accordance with the general literature data that increased methylation patterns of CDH1 seem to be involved in the initiation of tumorigenesis process. Apart from very limited CDH1 methylation studies conducted on salivary glands, oral cavity and head-neck tumors, to the best of our knowledge, there is yet no study investigating CDH1 methylation changes on parotid pleomorphic adenoma. In this study, CDH1 promoter methylation rate was 26.08 percent, relatively higher in parotid pleomorphic adenoma samples when compared with the methylation rate of normal parotid gland tissues (10%). Surely, it is an investigation issue of further studies whether CDH1 methylation strongly promotes the

development of parotid pleomorphic adenomas or not. The researchers do not draw firm conclusions related with CDH1 methylation's effect though a slight increase was observed. Multicentered studies removing the obstacle of the limited numbers used in this study (related with the rarity of this specific tumor type and material collection from one center) can deeply enlighten the effect of CDH1 methylation in the development of parotid neoplasms.

One of the other investigation subjects for researchers was the possible effect of HPV in parotid pleomorphic adenoma. HPV has a limited genetic repertoire of only eight ORFs but all the same it has capacity to modulate cellular pathways to its advantage. Cells infected with high-risk oncogenic HPV types are implicated as hyper-proliferative, anti-apoptotic and they lack of sufficient expression of markers required for immune detection (D'Costa et al. 2012). Some studies emphasize the role of HPV infection in the development of salivary gland diseases as in the study of Lin et al. (2014) though the researchers showed a significantly higher prevalence of HPV18 than HPV16 in pleomorphic adenomas in contrast to the results of this study. HPV infection was shown in a significant proportion of salivary gland tumors in the study of Hühns et al. (2015). Some studies on the other hand did not imply HPV as a causative agent of salivary gland tumors (Haeggblom et al. 2018). Therefore, more studies are definitely needed to draw more conclusive results. The researchers aimed to investigate the potential involvement of highly oncogenic viruses HPV16 and HPV18 in the development of parotid pleomorphic adenoma. Vageli et al. (2007) reported that 7 of 9 parotid lesions were HPV positive and 6 of these 7 positive samples had been infected by oncogenic types HPV16 and/or HPV18. The study of Descamps et al. (2012) did not support the prominent role of HPV infection in parotid carcinogenesis since only 3 samples out of 40 benign parotid tumors were HPV-positive. Teng et al. (2014) showed the association of HPV infection in parotid tumorigenesis since HPV positivity rates were 59.6 and 42.9 percent in benign and malignant parotid gland tumors, respectively while the infection rate was negative in normal oral mucosa tissue samples. One of our limitations related with HPV infection analysis was the use of only one consensus primer pair GP5+/GP6+ but not the other consensus primer pair MY09/MY11. As explained in detailed data in results part, the combined use of both consensus primers could be better especially in terms of the geographic location of the researchers' country since MY09/MY11 primer sets were used predominantly in studies in America and Asia while GP5+/GP6+ consensus primers were used mainly in Europe (Shikova et al. 2009). As a result, contradictory results in terms of the effect of HPV infection in the development of parotid lesions reflect the need of other studies in this field. In this relatively small study cohort, though HPV18 infection rate does not seem to play a role in parotid pleomorphic adenoma development since the ratios were 4.34 percent for tumors and 10 percent for control samples, HPV16 presence seems to be a partial risk factor in parotid tumorigenesis since all control samples were negative for this viral marker and 21.73 percent of the tumors were HPV-16 positive.

CONCLUSION

Specifically, HPV16 infection seems to be related with the development of parotid pleomorphic adenoma and this may reflect the importance of detection and quantification of human papillomavirus presence in parotid neoplasms. Promoter hypermethylation of CDH1 with a partial increased rate at parotid pleomorphic adenoma samples compared with the normal tissues may also be a recommendable study topic for future studies since individualized chemotherapies such as demethylating agents directed at this and/or other targets related with this gene could be developed.

RECOMMENDATIONS

To the best of of the researchers' knowledge, this is the first study evaluating the potential effect of CDH1 promoter methylation in parotid pleomorphic adenoma. Besides, the contradictory results related with the effect of HPV infection in the development of parotid lesions exist in literature. Therefore, this study's results could be beneficial to a certain extent to direct future researchers.Researchers' major limitation was the small sample cohort stemming from the rarity of

this specific tumor type. Therefore, researchers very strongly recommend multicentered studies reaching larger sample cohorts and thus drawing a more comprehensive picture on the effects of both CDH1 methylation and HPV infection in the development of parotid pleomorphic adenoma can be achieved.

ABBREVIATIONS

HPV: Human papilloma virus; FFPE: Formalin-fixed paraffin-embedded; PCR: Polymerase chain reaction; PA: Pleomorphic adenoma; CXPA: Carcinoma ex pleomorphic adenoma; MSP: Methylation specific PCR; SPSS: Statistical package for social science

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