Incidence of Genotypes of Helicobacter Pylori in Oropharyngeal SCC

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Abstract— Helicobacter pylori (HP) is a well known gastric pathogen which has been recently detected in the oral cavity and saliva an also in oropharynx, especially in adenoid and tonsillar tissue (Wladayer´s ring tissue). Presented research has been focused on real-time PCR analyzing cagA and vacA genotypes of HP strains in tonsills and oropharyngeal cancer.

Methods

The study was approved by the appropriate ethics committee.The tissue specimens of tonsillar cancer or tonsils were collected using sterile instruments at the beginning of surgery.Biopsies were immersed into Remel MicrotestR M4RT and transported to the laboratory for real-time PCR analysis. TaqManTM hybridisation probes were developed for cagA , , vacA m1 and vacA m2, LC hybridization probes for vacA s1a, vacA s1b, vacA s2 specific sequence detection.

Findings

Majority of isolates lacking cagA gene coding CagA protein as the main factor of Helicobacter pylori pathogenicity as well as one type of vacA gene s1b m2 found in 17 from a total of 41 isolates (41.46 %) and in 66.66 % of tonsillar cancer.

Interpretation

Colonization of lymphoid tissue and malignancies in oropharyngeal area by different genotypes of Helicobacter pylori seems to be evident. Long-term survival of bacteria in deep tissue structures may influence immune system.

Index Terms— Helicobacter pylori, oropharyngeal cancer, genotype

I. Introduction

Helicobacter pylori (HP) is a well known gastric pathogen. *Helicobacter pylori* is a declared type I carcinogen [1]. Since it was detected in the oral cavity [3,4] and orpharynx [5,6] a hypothesis about its possible influence in oropharyngeal pathogenesis is being considered [7,8].

HP is supposed to be spread from person to person by oral-oral or faecal-oral route [9]. Virulence of HP strains differs according to production of several virulence factors. The most important virulence factors, which were described as associated with gastric pathogenesis, are *CagA* (cytotoxic associated protein A) and *VacA* (vacuolizating cytotoxin A). Genome sequence analysis of *Helicobacter pylori* DNA led to identification of genes encoding these virulence factors

grouped in the so-called pathogenicity island (PAI) [10]. Specimens of 50 patients were collected and investigated: 35 with large spinocellular tumours of oropharynx (including 15 tonsillar, 4 of tongue base and 16 on the soft palate and walls of pharynx) and 15 with malignancies in hypopharyngeal and laryngeal area.

II. METHODS

Collection of Clinical Specimens

The study was approved by the appropriate ethics committee, and informed written consent was obtained from every participant. Indications for surgery were tonsillar spinocellular cancer (SCC).

The tissue specimens of tonsillar cancer or tonsils were collected using sterile instruments at the beginning of surgery, immediately after insertion of endotracheal tube, prior to application of local anaesthetics or disinfection substances into the oral cavity. Biopsies were immersed into Microtest^{R} M4RT transport media (Remel Inc., USA) and transported to the laboratory for real-time PCR analysis.

Preparation of Genomic DNA

HP DNA isolation was performed on MagNA Pure^{TM} Compact System (Tegimenta AG, Rotkreuz, Switzerland) using Protocol "Total_NA 400_100" and MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics) with pretreatment in MagNA Pure Bacteria Lysis Buffer (Roche Diagnostics). Isolated nucleic acid specimens were stored in -80°C until genotyping assays were performed.

PCR Amplification and Genotyping

For genotyping, 3 real-time PCR TaqMan^{TM} assays had been developed in cooperation with TIB-Molbiol Berlin, FRG, one for *cagA* gene, second for *vacA* gene middle region and the last one for *vacA* gene signal region. Primers used for PCR detection of HP were: cagA F (sense), cagA R (antisense), HPMGF+ (sense), HPMGR- (antisense), VAF1F+ (sense) and VAXR- (antisense) according to vanDoorn et al. 13

TaqMan^{TM} hybridisation probes were developed for *cagA* , *vacA* m1and *vacA* m2,and LC hybridization probes for *vacA* s1a, *vacA* s1b and *vacA* s2 specific sequence detection. For *cagA* assay FAM-BBQ labelled probe was used (detection 530 nm), for *vacA* middle region assay FAM-BBQ labelling was used for M1 probe (530 nm) and HEX-BBQ labelling for M2 probe (560 nm). TaqManR real-time PCR assays were run on LightCycler^{R} instrument, version 2.0 (six channel detection: 530, 570, 610, 640, 670 and 705 nm). Commercial LightCycler^{R} TaqMan^{TM} Master (Roche Applied Science) was used - 15 µl of MasterMix including primers and probes and 5 µl of sample DNA isolate per 20 µl capillary.

For real-time PCR for *vacA* gene signal region hybridisation probes S1a LC (LC610), S1b LC (LC640) and S2 LC (LC705) were used together with commercial LightCycler^{R} FastStart DNA MasterPLUS HybProbe (Roche Diagnostics). - 15 µl of MasterMix including primers and probes and 5 µl of sample DNA isolate per 20 µl capillary.

III. RESULTS

Data obtained in our study show evidence of Helicobacter pylori presence in the neoplastic tissue in oropharyngeal area, especially in lymphoid one. As there are not many recent studies to compare data with, we can conclude, that our significantly higher rate of detection (82%) compared with some studies performed in the first decade of 21st century is based on improved laboratory techniques and unigue selection of peroperative cancer tissue biopsies Tab.1.

Majority of isolates lacking *CagA* gene coding *CagA* protein as the main factor of *Helicobacter pylori* pathogenicity as well as one type of *VacA* gene s1b m2 found in 17 from a total of 41 isolates (41.46 %) and in 66.66 % of tonsillar cancer isolates Tab.2, may support hypothesis, that bacterium´s possible participation in cancerogenesis may lead via alteration of the host´s immune system mechanisms during long-term colonization in tissue, rather than by action of direct mutagen. Another important question could be asked on possible interaction of resident *Helicobacter pylori* with other infectious agents infecting tissue in this area, especially with DNA viruses with known oncogenic potential e.g. human papillomaviruses.

TABLE 1:
DETECTION OF HELICOBACTER PYLORI DNA IN TUMOUR SAMPLES FROM PATIENTS

Diagnosis	Number of samples	H.pylori detected	H. pylori not detected
Tonsillar cancer	15	12 80%	3 20%
Tongue base cancer	4	2 50%	2 50%
Cancer of soft palate and pharyngeal walls	16	12 75%	4 25%
Oropharyngeal cancer all locations	35	26 74.29%	9 25.71%
Hypo pharyngeal and Laryngeal cancer	15	15 100%	0
TOTAL	50	41 82%	9 18%

TABLE 2

DETECTION OF SPECIFIC SEQUENCIES OF H. PYLORI GENOMIC DNA ISOLATED FROM DIFFERENT TUMOUR SAMPLES

HYPOPHARYNGEAL CANCER ISOLATES	No. of samples	%
GENOTYPE : cagA+ vacA s1a m2	1	6.67
cagA- vacA s1a m2	3	20
cagA- vacA s1a m1	5	33.33
cagA- vacA s1b m2	5	33.33
cagA- vacA s2 m2	1	6.67
TOTAL	15	100

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OROPHARYNGEAL CANCER ISOLATES	No. of samples	%
GENOTYPE : cagA+ vacA s1a m2	3	11.54
cagA+ vacA s1b m2	1	3.84
cagA+ vacA s2 m1	1	3.84
cagA- vacA s1a m1	2	7.69
cagA- vacA s1a m2	2	7.69
cagA- vacA s1a m2	2	7.69
cagA- vacA s1b m2	11	42.32
cagA- vacA s1b m1	3	11.54
cagA- vacA s2 m2	3	11.54
TOTAL	26	100

PHARYNGEAL WALL AND SOFT PALATE	No. of samples	%
GENOTYPE : cagA+ vacA s1a m2	1	8.33
cagA- vacA s1a m2	1	8.33
cagA+ vacA s2 m1	1	8.33
cagA- vacA s2 m2	3	25
cagA- vacA s1b m2	2	16.67
cagA- vacA s1b m1	2	16.67
cagA- vacA s1a m1	2	16.67
TOTAL	12	100

TONSILLAR CANCER ISOLATES	No. of samples	%
GENOTYPE : cagA+ vacA s1b m2	1	8.34
cagA- vacA s1b m2	8	66.66
cagA+ vacA s1a m2	1	8.34
cagA- vacA s1a m2	1	8.34
cagA- vacA s1b m1	1	8.34
TOTAL	12	100

TONGUE BASE CANCER ISOLATES	No.of samples	%
GENOTYPE : cagA+ vacA s1a m2	1	50
cagA- vacA s1b m2	1	50
TOTAL	2	100

IV. DISSCUSION

The original assumption that oropharyngeal HP infection could participate in oropharyngeal carcinogenesis as a direct mutagen could not be confirmed [11,12]. An analogous situation occurs in the stomach [13-15]: although the HP seroprevalence is reportedly between 40-80% [16], serious stomach problems such as gastroduodenal ulcer disease or gastric cancer only develop in 10 -15% of infected people. Uemura et al. [17] performed a prospective study and found that gastric cancer developed in 36 of 1236 persons with gastric HP infection.

HP strain virulence varies depending on the production of several toxins, which is due to the presence of virulence factor genes, most importantly the *cagA* and *vacA* genes. The main carcinogenic effect of HP is in the presence of the *cagA* gene and the s1/m1 allele combination of the *vacA* gene [18]. A meta-analysis conducted by Huang et al. [19] showed that the risk of gastric cancer is twice as high in people who were positive for antibodies against CagA in sera. The analysis of oropharyngeal HP genotypes performed here showed increased abundance of the s1/m2 allele combination of alleles of the *vacA* gene. The *cagA* gene was found only in 5 (12.5%) cases. This finding means that less virulent HP strains predominated in the oropharynx. In comparison with the geographical epidemiological studies of gastric HP genotypes performed by Van Doorn et al. [20], the allele distribution of the *vacA* gene and the presence of the *cagA* gene differ considerably. The dominantly expressed signal sequence allele of the *vacA* gene in Europe is s1a (88.8%), and the dominant middle sequence allele is m1 (54.5%). The *cagA* gene was found in 72.1% of specimens. The results of our oropharyngeal genotype analysis, however, showed a majority of s1b (56.7%) and m2 (59.5%) alleles of the *vacA* gene and only the rare abundance of the *cagA* gene in oropharyngeal genotypes. The finding of an s2/m1 genotype can be considered to be very unique. The finding of this genotype was published in the literature only in isolated cases [21]. The finding of mixed genotypes of three HP strains is rare; however, it has been previously described [22]. Oropharyngeal tonsillar tissue is an extragastric reservoir of *Helicobacter pylori* infection [6, 23].

Multivariate regression analyses in two case control studies identified *Helicobacter pylori* infection as an independent risk factor for laryngohypopharyngeal carcinoma [24, 25]. A recent study of a large patient cohort associated immunohistochemically detected *Helicobacter pylori* expression in oral squamous cell carcinoma with reduced disease-free survival.

In contrast, others have not found *Helicobacter pylori* in head and neck cancers or in laryngeal carcinoma samples [6,26]. PCR, culture and immunohistochemical methods did not detect *Helicobacter pylori* in head and neck tumor tissues from 31 patients, even though 21 of them carried anti- Helicobacter pylori antibodies. A statistically significant difference in the incidence of *Helicobacter pylori* seropositivity between patients with head and neck cancer and controls has not yet been reported.

The presence of *Helicobacter pylori* in head and neck tumor tissues and/or the stomach of patients with head and neck malignancies might be widespread; however, more information is required about *Helicobacter pylori* activities in patients with head and neck carcinogenesis.

The oral cavity and/or tonsillar tissue, can be considered to be a possible extragastric reservoir of *Helicobacter pylori* infection [27, 28, 29]. Immunohistochemistry was used in several studies [30, 31]. PCR assay is to date considered the most appropriate method for detection of oropharyngeal *Helicobacter pylori*. This method was reported 100%

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sensitivity and specificity of detection of *Helicobacter pylori* [32]. In contrast, Hussey et al. [33] denied previous results of studies that had used PCR method for *Helicobacter pylori* detection in tonsillar tissue. In contrast to our study, Bonaventura et al. [34, 35] did not describe tonsillar tissue like an extragastric reservoir of *Helicobacter pylori*.

Helicobacter pylori stimulate Wnt/β-catenin pathway by activating oncogenic c-Met and epidermal growth factor receptor (EGFR), or by inhibiting tumor suppressor Runx3 and Trefoil factor 1 (TFF1). This facts described Song et al. [36]. *Helicobacter pylori* also trigger Wnt/β-catenin pathway by recruiting macrophages. Recently, by using gastroids, researchers have further revealed that *Helicobacter pylori* induce gastric epithelial cell proliferation through β-catenin. These findings indicate that Wnt/β-catenin is an oncogenic pathway activated by *Helicobacter pylori*. Therefore, this pathway is a potential therapy target for *Helicobacter pylori* -related gastric cancer. This pathway can be the new idea for next analyses by oropharyngeal cancer too.

Helicobacter pylori is able to survive the acidic gastric environment by producing the enzyme urease, which metabolizes urea to carbon dioxide and ammonia to buffer the gastric acid.

Helicobacter pylori moves across gastric mucus and can adhere to epithelial cells using a variety of adhesin-like proteins . [37]. Once adhered to epithelial cells, *Helicobacter pylori* induces a strong immune system response. This response does not lead to elimination of the bacterium, but causes development of chronic inflammation. *Helicobacter pylori* is not eradicated unless an infected individual is treated with a combination of antibiotics. Chemical products of *Helicobacter pylori* attract cells of the immune system into lamina propria [38]. It was shown that *Helicobacter pylori* can induce the maturation and activation of monocyte-derived dendritic cells. This activity is mediated by TLRs (Toll-like receptors) expressed on antigen presenting cells and leads to promotion of NK and Th1 effector responses [39]. IFN – gama producing Th1 polarized T cells and activated NK cells have been suggested to play an important role for development of severe pathologies [40].

V. Conclusions

Colonization of lymphoid tissue and malignancies in oropharyngeal area by different genotypes of *Helicobacter pylori* seems to be evident.

Bacteria *Helicobacter pylori* probably do not only adhere on superficial structures, but may invade tissues an persist there (similarly as in stomach).

Long-term survival of bacteria in deep tissue structures may influence immune system.

Mechanisms of immune response to such infection and interaction with bacterial virulence factors and defense mechanisms requires detailed investigation, as well as mechanisms of eventual bacterial participation in cancer development.

The main hypothesis are: Some infection agents modify local immunity in oropharyngeal region and “open” the door to the neoplasias and cancers. This agents modify (depress) the local immunity. The infection agents (*Helicobacter pylori*) start the Th1 and Th2 answer with overproduction of initiators of blocker of apoptosis, the modification of tyrosine kinase signal way.

Possibility of *Helicobacter pylori* cooperation with other infectious agents (e.g. *Fusobacterium*) occurring in this area, especially intracellular ones like viruses, should also be considered.

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