Original Article

HPV Status and Mutation Analysis Using Multiparallel Sequencing in Distal Oesophageal and Gastro-oesophageal Junction Adenocarcinomas

(gastro-oesophageal junction cancer / oesophageal cancer / adenocarcinoma / multiparallel sequencing / gene mutation / human papillomavirus)

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Abstract. The incidence of adenocarcinoma of oesophagus or gastro-oesophageal junction is increasing in Europe and other regions of the Western world. Research of possible causes has shifted to the molecular level. This study evaluated human papillomavirus (HPV) using real-time PCR and mutational status of selected genes using the multiparallel sequencing method (NGS) in DNA extracted from paraffin-embedded tumour tissue of 56 patients with oesophageal or gastro-oesophageal junction adenocarcinoma. The genetic material was in sufficient quality for the analysis in 37 cases (66 %). No HPV-positive sample was found. NGS revealed higher frequency of mutations in *TP53*, *ARID1A*, *PIK3CA*, *SMAD4*, *ERBB2*, *MSH6*, *BRCA2*, and *RET* genes. Association between gene mutations and histological grade, subtype according to Lauren, or primary tumour site was not statistically significant. In conclusion, the study did not confirm any HPV-positive sample of oesophageal and gastro-oesophageal junction adenocarcinoma. The study confirmed the usefulness of NGS analysis of paraffin-embedded tissue of these tumours, and it could be used in clinical studies to evaluate the prognostic and/or predictive value of the tested mutations. The association between gene mutations and histological features should be tested in larger patient cohorts.

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Abbreviations: AC – adenocarcinoma, ACMG – American College of Medical Genetics, HPV – human papillomavirus, GOJ – gastro-oesophageal junction, NGS – multiparallel sequencing method, SCC – squamous cell carcinoma, VAF – variant allele frequency, WES – whole-exome sequencing, WGS – whole-genome sequencing.

Introduction

Although the incidence of gastric adenocarcinoma and, similarly, oesophageal squamous cell carcinoma is decreasing in Europe and other Western regions, the incidence of adenocarcinoma (AC) arising from distal oesophagus or gastro-oesophageal junction (GOJ) still exhibits a rapidly increasing trend (Pennathur et al., 2013; Arnold et al., 2015). A universally acceptable explanation for this increase is still unknown. Risk factors for the development of oesophageal/GOJ AC under discussion include gastro-oesophageal reflux disease, Barrett's oesophagus, male sex, obesity and smoking (Rantanen et al., 2016; Coleman et al., 2018).

The search for possible causes has shifted to the molecular level. In addition, understanding the molecular pathogenesis could lead to widening the therapeutic options in oesophageal/GOJ AC. There are several studies

investigating specific molecular characteristics of oe-sophageal/GOJ AC, but the results are not conclusive. Mutational analyses detected genomic alterations in various genes, and among the most often mutated were *TP53, CDKN2A, ARID1A, PIK3CA, SMAD4*, and other (Dulak et al., 2013; Wang et al., 2015; Cancer Genome Atlas Reasearch Network, 2017). A recent study of Rajendra et al. (2016) demonstrated that mutational sig-natures differ between human papillomavirus (HPV)-positive and HPV-negative oesophageal AC, and ac-cording to the meta-analysis of Li et al. (2014), the probability of HPV positivity can be expected in ap-proximately 35 % of oesophageal ACs.

The primary objective of the present study was to evaluate the mutational status of candidate genes by multiparallel sequencing (NGS) of DNA obtained from distal oesophageal and gastro-oesophageal junction ad-enocarcinoma samples of patients, diagnosed and/or treated for this disease at the University Hospital Hradec Králové, with respect to the HPV status. The secondary objective was to verify a possible link between the his-tological subtype according to Lauren classification, histological grade and primary tumour location, and the mutational status of candidate genes.

Material and Methods

Fifty-six samples of distal oesophageal and GOJ AC, obtained from patients diagnosed and/or treated between 2000 and 2014, were available for the analysis. Primary endoscopic biopsies were analysed in 51 cases. In five cases, only samples from definitive surgery after neoad-juvant treatment were available. Based on the results reported by Noorani et al. (2017) showing no significant differences in overall mutation rate or mutational signa-tures based on whole-genome sequencing analysis be-tween samples obtained before and after chemotherapy, we decided to include these samples in the analysis.

Anonymized tumour samples were analysed accord-ing to the protocol. The DNA was extracted from paraf-fin-embedded tissue blocks by the Cobas DNA Sample Preparation Kit (Roche, Mannheim, Germany) accord-ing to the manufacturer's protocol.

HPV DNA detection was performed by real-time PCR with the AmoyDx Human Papillomavirus Geno-typing Detection Kit (Amoy Diagnostic Co., Fuijan, China) according to the manufacturer's protocol. The test is designed for detection and genotyping of 19 high-risk HPVs (HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82) and two low-risk HPVs (HPV6 and 11).

Mutation analysis was performed by multiparallel se-quencing. Indexed Illumina NGS library was construct-ed from 100 ng tumour DNA by a KAPA Library Preparation Hyper Plus Kit (Kapa Biosystems, Roche). Hybrid selection was performed with a custom SeqCap EZ Choice Library (Roche NimbleGen). The library was designed using genome build hg19 NCBI Build 37.1/GRCh37.

Genes in the sequencing panel included (transcript ref-erence numbers are in brackets) *ARID1A* (NM_006015), *BRCA1* (NM_007294), *BRCA2* (NM_000059), *CDKN2A* (NM_000077), *CTNNB1* (NM_001904), *EGFR* (NM_005228), *EPHB2-ERK* (NM_004442), *ERBB2* (NM_004448), *ESR1* (NM_000125), *FBXW7* (NM_033632), *FOXL2* (NM_023067), *GNA11* (NM_002067), *GNAQ* (NM_002072), *HRAS* (NM_176795), *KIT* (NM_000222), *KRAS* (NM_004985), *MDM2* (NM_002392), *MET* (NM_001127500), *MLH1* (NM_000249), *MSH2* (NM_000251), *MSH6* (NM_000179), NOTCH1 (NM_017617), NRAS (NM_002524), *PDGFRA* (NM_006206), *PIK3CA* (NM_006218), *PMS2* (NM_000535), *PTEN* (NM_000314), *SMARCA4* (NM_001128844), *SMARCB1* (NM_003073), *TP53* (NM_000546), *ALK* (NM_004304), *APC* (NM_000038), *FGFR2* (NM_000141), *MAP2K1* (NM_002755), *CDH1* (NM_004360), *SMAD4* (NM_005359), *STK11* (NM_000455), *SRC* (NM_005417), *GNAS* (NM_000516), *IDH1* (NM_005896), and *IDH2* (NM_002168).

Paired-end cluster generation and sequencing was performed according to the standard protocols using v2 kits (Illumina Inc,, San Diego, CA). Sequencing data analysis and variant classification was performed by NextGENe software (Softgenetics, State College, PA) with 10 % and 5 % variant allele frequency (VAF) filter-ing, respectively. VAF frequencies of 10 % and 5 % were used as both these levels are considered to be clin-ically relevant (Strom, 2016). A variant allele was re-ported as mutated in case of grade 4 or 5 according to the ACMG (American College of Medical Genetics) pathogenicity scale.

For statistical analysis, χ^2 test and Fisher's exact test were used to verify the statistical link between the muta-tional status of candidate genes and histological sub-type, grade and primary tumour location. Bonferroni correction was used to counteract the problem of multi-ple comparisons. Logistic regression analysis was used to determine the influence of archiving time on genetic analysis failure. We considered $P < 0.05$ to be statisti-cally significant. All statistical analyses were performed using the NCSS 8 statistical software program (NCSS, Keysville, UT).

Results

Fifty-six samples of distal oesophageal (28 samples) and GOJ AC (28 samples) obtained from patients diag-nosed and/or treated between 2000 and 2014 were iden-tified. Forty-eight of these patients (84 %) were male and the median age was 63 years (range 36–75 years). Most of the patients (N = 51; 91 %) were treated for lo-cally or regionally advanced disease (T3-4 or N+ M0). In one case the patient had distal metastases, in two cases tumours were in early stage, and two patients had incomplete staging evaluation and definitive staging could not be defined.

In 51 cases, endoscopically obtained biopsies (91 %), and in five cases, only samples from definitive surgery after neoadjuvant treatment were available. The genomic analysis was not successful in 19 of 56 samples (34 %) because of DNA damage. The cause of the failure was a long archiving time of the samples. The effect of archiving time on genetic analysis failure was confirmed by logistic regression analysis (odds ratio (OR) 1.43; 95% confidence interval (CI) 1.17–1.74; P = 0.0005).

Therefore, only 37 samples (66 %) were included in the statistical analysis. Nine of these samples were of diffuse or mixed subtype and 28 were of intestinal subtype of AC according to Lauren. Four, 13 and 20 samples were classified as histological grade 1, grade 2 and grade 3, respectively.

The analysis did not detect any HPV-positive sample, and all samples were therefore classified as HPV negative.

Mutations were detected only in *MLH1*, *MSH6*, *TP53*, *ARID1*, *ERBB2*, *SMAD4*, *BRCA1*, *BRCA2*, *PTEN*, *RET*, *PDGFRA*, *PIK3CA*, *APC*, *KRAS*, *GNAS*, *CDKN2A*, *MET*, and *GNAQ* genes. In the remaining genes, a mutation of grade 4 or 5 according to the ACMG pathogenicity scale was not found with a minimum of 5% variant allele frequency (VAF) filtering.

Mutations were found the most frequently in the *TP53* gene, with the rate of 49 % and 59 % for VAF cutoff of 10 % and 5 %, respectively. Except for the *TP53* gene, a higher rate of mutation was detected in *PIK3CA*, *ERBB2*, *MSH6*, and *RET* genes with the VAF cut-off of 10 %. When the cut-off level was lowered to 5 %, higher mutation probability was also found in *BRCA2* and *ARID1* genes. Gene mutation frequencies are shown in detail in Table 1.

Subsequent analysis was focused on mutation types in the genes of interest. In summary, nonsense mutations were quite rare (rate ≤ 5%). With 5% VAF filtering, the occurrence of missense and frameshift mutations were comparable (49 % and 46 %, respectively). With 10% VAF filtering, missense mutations were more common (60 % versus 35 %). When individual genes were analysed, missense mutations were unequivocally predominant in the *TP53* gene: 72 % and 77 % for 10% and 5% VAF filtering, respectively. In contrast, all nine mutations were frameshift in *MSH6*, the second most frequent mutated gene with 5% VAF filtering. The mutation type sub-analysis is reported in detail in Tables 2 and 3.

The data obtained were used for evaluation of the possible association of gene mutational status and histological subtype of AC (according to Lauren classification), histological grade or primary tumour site origin. The statistical analysis found associations of *MSH6* mutation with diffuse or mixed subtype according to Lauren classification (VAF cut-off 10 %; χ^2 test P = 0.012, Fisher exact test P = 0.037) and *PIK3CA* mutation with distal oesophagus as the primary site (VAF cut-off 10 %; χ^2 test P = 0.027, Fisher's exact test P = 0.049), but these results were not statistically significant when the Bonferroni correction for multiple tests was used.

Discussion

The present study evaluated the mutational status of 41 genes in DNA extracted from paraffin-embedded oe-

Table 1. Detected variant allele frequency

Gene	Variant allele frequency 10% cut-off (%)	Variant allele frequency 5% cut-off (%)
MLH1	0	3
MSH6	11	24
TP53	49	59
ARID1	5	16
ERBB2	13	19
SMAD4	5	5
BRCA1	3	5
BRCA2	3	16
PTEN	0	5
RET	11	16
PIK3CA	13	19
APC	3	5
KRAS	3	3
GNAS	0	3
CDKN2A	5	5
MET	5	5
GNAQ	0	3
PDGFRA	0	3

Table 2. Absolute number of mutation types in genes of interest with 10% variant allele frequency filtering. The table encompasses only genes in which a mutation was detected in at least one patient.

Gene	Missense	Nonsense	Frameshift	All
MSH6			4	4
TP53	13	2	3	18
ARID1			2	2
ERBB2	3		2	5
SMAD4	1		1	2
BRCA1	1			1
BRCA2	1			1
RET	3		1	4
PIK3CA	5			5
APC			1	1
KRAS	1			1
CDKN2A	1		1	2
MET			2	2
Absolute number	**29**	**2**	**17**	**48**
Relative number	**60.4 %**	**4.2 %**	**35.4 %**	**100 %**

Table 3. Absolute number of mutation types in genes of interest with 5% variant allele frequency filtering. The table encompasses only genes in which a mutation was detected in at least one patient.

Gene	Missense	Nonsense	Frameshift	All
MLH1		1		1
MSH6			9	9
TP53	17	2	3	22
ARID1	1		5	6
ERBB2	3		4	7
SMAD4	1		1	2
BRCA1	1		1	2
BRCA2	1	1	4	6
PTEN			2	2
RET	4		2	6
PIK3CA	7			7
APC			2	2
KRAS	1			1
GNAS	1			1
CDKN2A	1		1	2
MET			2	2
GNAQ			1	1
PDGFRA	1			1
Absolute number	**39**	**4**	**37**	**80**
Relative number	**48.7 %**	**5 %**	**46.2 %**	**100 %**

sophageal or GOJ adenocarcinomas, using the multiparallel sequencing method. Recently, Rajendra et al. (2016) have demonstrated that mutational signatures differ between HPV-positive and HPV-negative oesophageal AC. The meta-analysis of HPV prevalence in oesophageal cancer found substantial differences among previously reported studies (1–90 %), although these studies had limited numbers of patients (in total 174 patients; Li et al., 2014). However, based on these data, the meta-analysis estimated the prevalence of HPV positivity in oesophageal AC of 35 %. Therefore, the present study started the analysis with HPV status evaluation. The study found no HPV-positive tumour sample. This result, described in the Czech population, corresponds to the results of a recent Australian study (Antonsson et al., 2016) that did not detect any HPV positivity among 233 histologically confirmed archived ACs of oesophagus or gastro-oesophageal junction.

Several studies describing the mutational status of oesophageal/GOJ ACs have been published. However, the largest studies used the whole-exome sequencing (WES) and/or whole-genome sequencing (WGS) method. Dulak et al. (2013) analysed 149 samples of oesophageal ACs using WES, with WGS performed in 15 of these samples, and confirmed previously reported higher frequency of mutations in TP53, CDKN2A, SMAD4, ARID1A, and PIK3CA, and also described other muta-

tions not previously reported in oesophageal AC in SPG20, TLR4, ELMO1, and DOCK2 genes. Similarly, the study recently published by the Cancer Genome Atlas Research Network (2017) included, except oesophageal squamous cell carcinoma (SCC) and undifferrentiated carcinomas, seven oesophageal ACs, 164 GOJ ACs and 141 samples of AC of distal stomach. This large and important study used WES and/or WGS and DNA methylation analysis, somatic copy number analysis, low-pass WGS for rearrangement genome identification, miRNA sequencing, reverse phase protein array, and microbiome analysis. The study revealed substantial differences between oesophageal SCC and AC. In oesophageal AC, mutations were most frequent in TP53, CDKN2A, ARID1A, SMAD4, and ERBB2 genes.

Generally, mutations in TP53 and CDKN2A are considered to be the most frequent mutations in oesophageal AC. TP53 mutation frequency is reported in 70–83 % cases (Chung et al., 2007; Dulak et al., 2013; Wang et al., 2015; Cancer Genome Atlas Reasearch Network, 2017), but is probably lower in GOJ AC. In the study reported by Li-Chang et al. (2015), TP53 mutations were identified in 42 % of GOJ tumours and 27 % of gastric tumours. The present study identified TP53 mutations in 59 % cases (cut-off 5 %), but this study included tumours from both, distal oesophagus and GOJ. For oesophageal AC alone or GOJ AC alone in the present study, the frequency of TP53 mutations was 70 % and 53 %, respectively. This supplementary data confirms a higher probability of TP53 mutation in oesophagus compared to GOJ.

CDKN2A mutations were identified only in 5 % of cases in the present study. Although this frequency seems lower than in previous studies (12–14 %) (Dulak et al., 2013; Cancer Genome Atlas Reasearch Network, 2017), we cannot consider it different because of the small number of events. Furthermore, in case of the CDKN2A gene, somatic mutations are apparently not so frequent compared to TP53. The frequency of CDKN2A alterations was even 81 % in the Cancer Genome Atlas Research Network study, but most of these alterations were epigenetic.

In contrast, the present study revealed higher frequency of mutations of PIK3CA (19 %) and ERBB2 (19 %) genes compared to previous studies reporting frequencies of 6 % (Cancer Genome Atlas Research Network, 2017) and 3–14 %, respectively (Dulak et al., 2013; Cancer Genome Atlas Reasearch Network, 2017). The present results are more comparable with the study of Wang et al. (2015) that used the NGS method for the analysis and reported mutation frequencies in PIK3CA and ERBB2 genes of 10 % and 23 %, respectively. On the other hand, that study has a much higher frequency of mutations of the KRAS gene (23 % compared to 3 % in the present study).

Unexpectedly, the present study found a higher frequency of mutations in MSH6 (but not in other mismatch-repair genes) and RET genes. It must be mentioned that in the case of MSH6 mutations, most were

with the allele frequency less than 10 % and all were frameshift mutations.

Generally, in terms of frequency of mutations found in candidate genes, the present study both partly confirmed the results of previous studies and showed some differences. An important finding is the confirmation that NGS analysis of DNA extracted from paraffin-embedded tumour tissue is suitable for retrospective analyses of patient cohorts, although the success of the analysis decreases with longer time of sample archiving.

Further analyses tested a possible association of the gene mutational status and histological characteristics of AC (grading or subtype according to Lauren), which has not been studied so far in oesophageal and GOJ AC, and primary tumour site. Although this analysis found an association of *MSH6* mutation with diffuse or mixed subtype according to Lauren classification (χ^2 test P = 0.012) and *PIK3CA* mutation with distal oesophagus as a tumour origin (χ^2 test P = 0.027), these results were not statistically significant when Bonferroni correction for multiple tests was used. Thus, these findings should be regarded as exploratory and tested in larger cohorts.

In conclusion, the present study did not confirm any HPV-positive sample of oesophageal/GOJ adenocarcinoma in the Czech population cohort. In terms of genetic analysis, generally, the study confirmed the usefulness of NGS analysis of paraffin-embedded tumour tissue of these tumours, although there is a risk of unsuccessful analysis if the sample is not of sufficient quality. We are aware of the disadvantages, mainly including a necessity of optimal gene library that should be prepared based on the results of WES and WGS methods. Furthermore, NGS does not evaluate epigenetic changes. This is relevant for example in *CDKN2A* gene analysis, as it can be supposed that DNA methylations are more frequent than genetic changes. On the other hand, NGS is a more accessible and cheaper method in comparison to WES and WGS, and the results of the present study partly showed similar results compared to the large studies using WES and/or WGS methods. Therefore, NGS could be used more easily for clinical studies that evaluate the prognostic and/or predictive value of these mutations. The results of the present study can serve to select genes and define a protocol for these studies.

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