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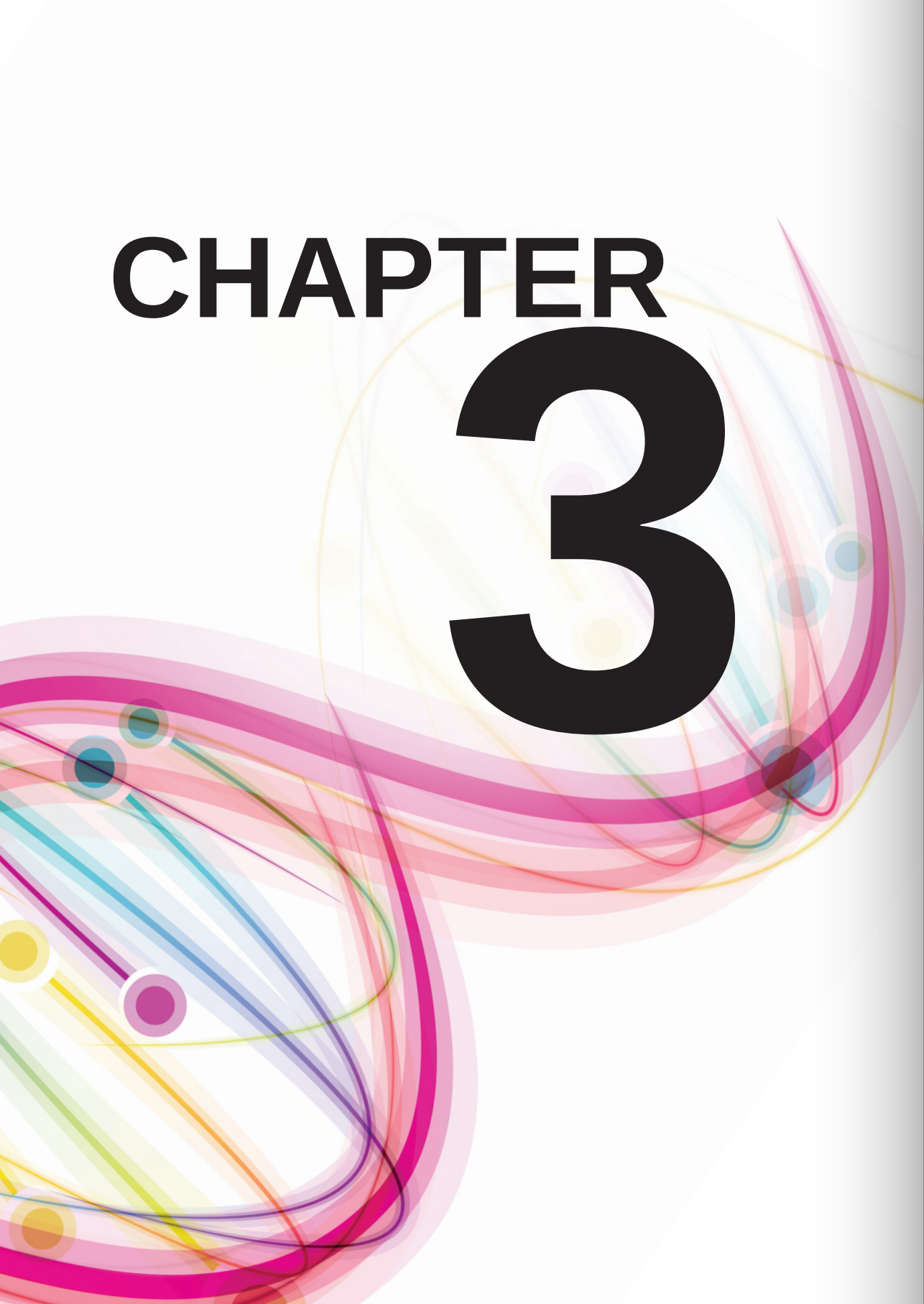
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CHAPTER 3

Somatic mutation in *PIK3CA*
is a late event in cervical
carcinogenesis

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ABSTRACT

Somatic mutations in cervical intraepithelial neoplasia (CIN) are largely unknown. Here, we profiled 35 cervical carcinomas and 23 CIN grade 2/3 (CIN2/3) for mutations in 48 cancer-related genes using a next generation sequencing-based cancer panel. *PIK3CA* exon 9 was the most frequently mutated locus in cervical carcinoma and the only mutated locus detected in CIN2/3. These *PIK3CA* exon 9 mutation findings were verified in a large, independent series (n = 647) covering all stages of cervical carcinogenesis using high resolution melting-guided Sanger sequencing. *PIK3CA* exon 9 mutation frequency was 37.1% (13/35; 95%CI 21.2-54.0%) in cervical carcinoma and 2.4% (5/209; 95%CI 0.5-4.7%) in CIN3. No *PIK3CA* exon 9 mutations were detected in CIN2 (0/144), CIN1 (0/154) and normal cervix (0/105). In a third series of 46 CIN2/3 lesions from women with a known 5-year history of preceding high-risk human papillomavirus (hrHPV) infection, detection of *PIK3CA* exon 9 mutation was confined to 2 (5.4%; 95%CI 0.0-13.2%) CIN3 lesions with preceding hrHPV infection ≥ 5 years, and was absent in those with a short duration (< 5 years) of preceding hrHPV infection. In conclusion, somatic mutation in *PIK3CA* represents a late event during cervical carcinogenesis, detected in a substantial subset of cervical carcinoma, but only in a minority of CIN3.

INTRODUCTION

A persistent infection with high-risk human papillomavirus (hrHPV) is the necessary cause of cervical cancer, yet additional genetic and epigenetic host cell aberrations are required for progression to invasive cancer¹. To date, genomic studies on cervical cancer and its squamous precursor lesions, cervical intraepithelial neoplasia (CIN) grade 1-3, have mostly focused on chromosomal aberrations and epigenetic features^{1,2}. A few studies, mainly restricted to cervical cancer, have concentrated on somatic mutations. These studies have revealed *PIK3CA* as the most frequently mutated gene with frequencies of 5.7-35.7%³⁻¹¹. So far, CIN lesions have not been subjected to mutation analysis in a comprehensive manner. It therefore remains unclear at what stage during cervical carcinogenesis somatic mutations occur. In this study, we perform comprehensive mutation profiling of squamous precursor lesions of cervical cancer.

MATERIALS AND METHODS

Clinical specimens

Frozen tissue specimens of cervical squamous cell carcinoma (SCC, $n = 25$), cervical adenocarcinoma (AdCA, $n = 10$) and CIN2/3 ($n = 23$) were used for mutation profiling of 48 cancer-related genes using a next generation sequencing (NGS)-based cancer panel (further referred to as TSACP-MiSeq-NGS). Given limitations in accurate histological grading of high-grade CIN on frozen tissue sections, CIN2 and CIN3 were grouped together in the frozen tissue sample set. An independent series of formalin-fixed paraffin-embedded (FFPE) tissue specimens covering all stages of cervical carcinogenesis was used for verification studies by high resolution melting-guided Sanger sequencing. This series consisted of cervical SCC ($n = 28$), cervical AdCA ($n = 7$), CIN3 ($n = 209$), CIN2 ($n = 144$), CIN1 ($n = 154$) and normal cervical epithelium ($n = 105$). An additional FFPE series of CIN2 ($n = 3$) and CIN3 ($n = 43$) was from women participating in the Population Based Screening Study Amsterdam (POBASCAM) and detected in the second screening round (interval 5 years) in the control arm (cytology screening with blind hrHPV testing)¹². Accordingly, the 5-year history of hrHPV infection of these women was known. The duration of preceding hrHPV infection (PHI) was considered as a proxy for duration of lesion existence¹³. Women with the same hrHPV type in their biopsy compared with baseline have PHI ≥ 5 years and were considered to have more advanced lesions (3 CIN2 and 34 CIN3). Women who acquired the hrHPV infection after study entrance (PHI < 5 years) were considered to have early lesions (9 CIN3). The study followed the ethical guidelines of VU University Medical Centre.

TSACP-MiSeq-NGS and *PIK3CA* exon 9 HRM-sequencing

Genomic DNA was isolated by Qiagen DNA Micro kit (Qiagen, Hilden, Germany) or easyMAG (Biomérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Genomic DNA from frozen tissue specimens was subjected to amplicon-based NGS using TruSeq Amplicon Cancer Panel (TSACP; Illumina, San Diego, CA, USA) and the MiSeq Personal Sequencer (Illumina), essentially as described before¹⁴. TSACP covers 212 amplicons from 48 cancer-related genes, which are simultaneously amplified in a single-tube reaction. A minimal variant allele frequency (VAF) of 0.05 was used to score mutations. High-resolution melting followed by Sanger sequencing (HRM-sequencing) for *PIK3CA* exon 9 was applied to genomic DNA from FFPE-tissue specimens, as previously described¹⁴.

Statistical analysis

Mutation frequencies were calculated with 95% confidence intervals (CI). Differences between groups were analysed by Chi-square test using cross-tabulation. A P value below 0.05 was considered statistically significant. Statistical analysis was performed with IBM SPSS v20.

RESULTS

Mutation profiling of cervical carcinoma and CIN2/3

To evaluate the presence of somatic hotspot mutations in 48 cancer-related genes, a NGS-based panel (TSACP-MiSeq-NGS) was applied to 25 SCC, 10 AdCA and 23 CIN2/3 (Table 1). Sixteen tumours (45.7%; 95%CI 28.6-62.9%) showed one or more non-synonymous mutations. Overall mutation frequency was comparable between SCC (12/25; 48.0%; 95%CI 27.6-68.0%) and AdCA (4/10; 40.0%; 95%CI 11.1-71.4%; $P = 0.668$). Most tumours harboured a single mutation (13/16; 81.3%). Co-occurrence of mutations was found in three tumours: *PIK3CA* and *FBXW7* (1 SCC), *PIK3CA* and *TP53* (1 SCC), and *PTEN* and *ATM* (1 AdCA). *PIK3CA* exon 9 was the most frequently mutated locus in cervical carcinoma (overall: 7/35; 20.0%; 95%CI 8.0-34.1%; SCC: 6/25; 24.0%; 95%CI 8.3-43.8% and AdCA: 1/10; 10.0%; 95%CI 0.0-33.3%) with median VAF of 0.23 (range 0.09-0.63). All *PIK3CA* mutations involved hotspot sites in exon 9, i.e. codon 542 (2/7; 28.6%) and codon 545 (5/7; 71.4%). Only one CIN2/3 (4.3%; 95%CI 0.0-14.3%) harboured a mutation, concerning *PIK3CA* p.E542K (VAF of 0.05).

Table 1. Mutations in cervical carcinoma and CIN2/3 as assessed by TSACP-MiSeq-NGS.

Gene	Mutation		CIN2/3 (n=23) n (VAF)	SCC (n=25) n (VAF)	AdCA (n=10) n (VAF)
	CDS change	Amino acid change			
<i>PIK3CA</i>	c.1624G>A	p.E542K	1 (0.05)	2 (0.13, 0.61)	0
	c.1633G>A	p.E545K	0	4 (0.09 ¹ , 0.14, 0.23, 0.32 ²)	0
	c.1633G>C	p.E545Q	0	0	1 (0.63)
<i>FBXW7</i>	c.1273C>G	p.R425G	0	2 (0.14 ¹ , 0.16)	0
<i>PTEN</i>	c.65A>G	p.D22G	0	1 (0.39)	0
	c.724G>T	p.E242*	0	0	1 (0.71 ³)
<i>APC</i>	c.3400G>C	p.D1134H	0	1 (0.30)	0
<i>STK11</i>	c.597G>C	p.E199D	0	1 (0.23)	0
<i>AKT1</i>	c.82C>G	p.L28V	0	1 (0.11)	0
<i>RB1</i>	IVS3 -1 G>C	Acceptor splice site	0	1 (0.57)	0
<i>TP53</i>	c.586C>T	p.R196*	0	1 (0.06 ²)	0
<i>NRAS</i>	c.182A>G	p.Q61R	0	0	1 (0.30)
<i>GNA11</i>	c.972C>G	p.I324M	0	0	1 (0.15)
<i>ATM</i>	c.7322T>G	p.V2441G	0	0	1 (0.50 ³)

AdCA: adenocarcinoma; CDS: coding DNA sequence; CIN: cervical intraepithelial neoplasia; SCC: squamous cell carcinoma; TSACP-MiSeq-NGS: TruSeq Amplicon Cancer Panel-based next generation sequencing on the MiSeq Personal Sequencer; VAF: variant allele frequency depicted for each mutated specimen separately.
^{1,2,3} Co-occurrence of mutations (number indicating the combination).

PIK3CA exon 9 mutations in an independent set covering all stages of cervical carcinogenesis

Given that *PIK3CA* exon 9 was the most frequently mutated locus, further mutation scanning was performed by HRM-sequencing spanning that locus. Testing of the above series by HRM-sequencing verified the TSACP-MiSeq-NGS results for *PIK3CA* exon 9. Next, a large independent series comprising 28 SCC, 7 AdCA, 209 CIN3, 144 CIN2, 154 CIN1 and 105 normal cervical epithelium, was evaluated to investigate the onset of *PIK3CA* exon 9 mutations during cervical cancer development (Table 2A). *PIK3CA* exon 9 mutations were detected in 13 cervical cancers (37.1%; 95%CI 21.2-54.0%), including 12 SCC (42.9%; 95%CI 23.8-62.5%) and 1 AdCA (14.3%; 95%CI 0.0-50.0%). Mutation frequency tended to be higher in SCC, but the difference between the two histological types was not significant ($P = 0.162$). Most mutations involved hotspots at codon 542 (6/13; 46.0%) or 545 (6/13; 46.0%). Five CIN3 lesions (2.4%; 95%CI 0.5-4.7%) showed a *PIK3CA* exon 9 hotspot mutation, i.e. p.E542K (1/5; 20.0%) and p.E545K (4/5; 80.0%). *PIK3CA* exon 9 mutations were not found in CIN2, CIN1 and normal cervical epithelium.

Table 2. Overview of *PIK3CA* exon 9 mutations in cervical tissue specimens as assessed by HRM-sequencing.

A. Cross-sectional series stratified by histological grade								B. CIN2 and CIN3 stratified by known duration of preceding hrHPV infection	
<i>PIK3CA</i> exon 9 mutation		Normal	CIN1	CIN2	CIN3	SCC	AdCA	Early	Advanced
CDS	Amino acid	(n=105)	(n=154)	(n=144)	(n=209)	(n=28)	(n=7)	(n=9)	(n=37)
change	change	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
c.1624G>A	p.E542K	0 (0)	0 (0)	0 (0)	1 (0.5)	5 (17.9)	0 (0)	0 (0)	0 (0)
c.1624G>C	p.E542Q	0 (0)	0 (0)	0 (0)	0 (0)	1 (3.6)	0 (0)	0 (0)	0 (0)
c.1633G>A	p.E545K	0 (0)	0 (0)	0 (0)	4 (1.9)	5 (17.9)	1 (14.3)	0 (0)	2 (5.4)
c.1637A>G	p.Q546R	0 (0)	0 (0)	0 (0)	0 (0)	1 (3.6)	0 (0)	0 (0)	0 (0)
Overall		0 (0)	0 (0)	0 (0)	5 (2.4)	12 (42.9)	1 (14.3)	0 (0)	2 (5.4)

AdCA: adenocarcinoma; Advanced: CIN2 (n=3) and CIN3 (n=34) from women with a preceding hrHPV infection ≥ 5 years; CDS: coding DNA sequence; CIN: cervical intraepithelial neoplasia; Early: CIN3 from women with a preceding hrHPV infection < 5 years; SCC: squamous cell carcinoma.

***PIK3CA* exon 9 mutations in CIN2 and CIN3 with different durations of lesion existence**

Finally, we assessed the occurrence of *PIK3CA* exon 9 mutations in relation to duration of lesion existence. To this end, we analysed an independent series of CIN2 and CIN3 diagnosed in women participating in the POBASCAM trial with a known 5-year history of preceding hrHPV infection¹³. These comprised early (n = 9) lesions from women with a known preceding hrHPV infection of < 5 years and advanced (n = 37) lesions from women with a preceding hrHPV infection lasting ≥ 5 years as a proxy of longer duration of lesion existence. Two advanced CIN3 lesions (5.4%; 95%CI 0.0-13.2%) harboured a *PIK3CA* (p.E545K) mutation, but none of the early lesions (Table 2B).

DISCUSSION

This study shows that somatic mutations in *PIK3CA* represent a late event during cervical carcinogenesis. A substantial subset of cervical carcinomas harboured a *PIK3CA* exon 9 mutation, but only a minority of CIN3, and no CIN2 or CIN1. The low VAF of *PIK3CA* exon 9 mutation in cervical cancer suggests that it is present in subclones and further supports this being a late event in tumour evolution. In a limited number of high-grade CIN with known duration of preceding hrHPV infection, *PIK3CA* mutations were only present in so-called advanced CIN3 and at low prevalence. By combining the current findings with those from our previous studies, we may infer the timing of different (epi)genetic aberrations following the initiating event (i.e. a transforming hrHPV infection) during cervical carcinogenesis. Relative to chromosomal aberrations and altered DNA methylation, which are detectable in the great majority of cervical carcinomas and advanced CIN3^{1,13,15,16}, *PIK3CA* mutations can be considered a late event.

To the best of our knowledge, this is the first study evaluating large series of CIN for somatic mutations. Mutations in cancer-related genes were present in almost half of cervical carcinomas. The finding that *PIK3CA* is the most frequently mutated gene in cervical cancers is in line with previous reports³⁻¹¹. However, mutations in cancer-related genes appeared to be rare in CIN lesions. To date, no *PIK3CA* mutations have been reported in CIN, but earlier studies comprised limited sample series^{5,8}.

A limitation of our study might be seen in the targeted NGS approach. Mutations outside the targeted genomic regions thereby remain undetected, with potential underrepresentation of overall mutation frequency as a result. Furthermore, the technology may be restricted in the identification of subclonal mutations that might be present at a VAF below the detection threshold of the assay. In addition, no normal DNA was available to verify the somatic origin of the mutations identified.

Of the *PIK3CA* mutations found, the only mutated locus in this gene was exon 9. In fact, the majority of these *PIK3CA* mutations (25/28; 89.3%) were hotspot mutations p.E542K (c.1624G>A) and p.E545K (c.1633G>A). These alterations both correspond to C > T (i.e. G > A on the opposing strand) mutations at a TCW (W = A or T) trinucleotide motif. Previously, a significant skew towards TCW mutations in *PIK3CA* was found in hrHPV-positive head and neck SCC, suggesting involvement of apolipoprotein B mRNA editing enzyme (APOBEC)¹⁷. These enzymes are normally involved in deamination of cytosines, which results in conversion to uracil, during RNA editing. Replacement of uracil in the DNA generally results in mutations to thymine or guanine^{17,18}. A role for APOBEC in response to hrHPV infection

has been proposed as a host defence mechanism and is hypothesised to trigger APOBEC signature mutations in hrHPV-associated cancers¹⁹.

Apart from mutations at hotspot codons 542 and 545, p.Q546R was present in one cervical carcinoma. The latter has not previously been described in cervical cancer, but has been reported in other cancer types, such as colon cancer²⁰. Mutations in other genes were rarely identified in cervical carcinoma. The second most mutated gene in SCC was *FBXW7*, with a frequency (8.0%; 95%CI 0.0-20.0%) in-between previously published rates (1.5-15.0%), but these concerned other base substitutions^{4,7}.

In conclusion, *PIK3CA* exon 9 mutations occur in a substantial subset of cervical cancer, while rarely in CIN3, and not in lower grade CIN nor normal cervix. Our data support that *PIK3CA* mutations represent a late event during cervical carcinogenesis.

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