Gynecologic Oncology 165 (2022) 121–128

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

Driver gene mutations in micro-invasive cervical squamous cancers have no prognostic significance

Karl Kashofer [a], Sigrid Regauer [a,*], Olaf Reich [b], Edgar Petru [b], Elke Winter [a]

[a] Diagnostic- and Research Institute of Pathology, Medical University Graz, Neue Stiftingtalstrasse 6, 8010 Graz, Austria
[b] Department of Gynecology and Obstetrics, Medical University Graz, Auenbruggerplatz 14, 8036 Graz, Austria

HIGHLIGHTS

- In cervical carcinogenesis somatic driver gene mutations occur as early as in micro-invasive pT1a1 SCC.
- PIK3CA driver gene mutations dominate in early invasive mutated SCC at frequencies comparable to advanced SCC.
- The growth advantage of tumor cells with driver gene mutations increases with the length of undetected tumor growth.
- Early detection of SCC at a micro-invasive stage followed by surgical removal of all tumor cells cures a patient.
- In contrast to advanced inoperable SCC, mutational status in surgically treated pT1a SCC has no prognostic value.

ARTICLE INFO

Article history:
Received 16 December 2021
Received in revised form 12 January 2022
Accepted 14 January 2022
Available online 31 January 2022

Keywords:
Cancer hot spot panel
Cervical carcinogenesis
PIK3CA gene
Somatic gene mutation
Chromosomal copy number variations
pT1a SCC

ABSTRACT

Objective. To evaluate the prevalence of somatic gene mutations in different stages of cervical carcinogenesis placing special emphasis on micro-invasive pT1a cervical squamous cell cancers (SCC).

Methods. Micro-dissected samples of 32 micro-invasive pT1a and 55 ≥ pT1b SCC were evaluated by next generation sequencing of 50 cancer genes (cancer hot spot panel).

Results. At primary diagnosis, 8/32 (25%) pT1a SCC, 10/28 (36%) pT1b SCC and 15/27 (56%) pT2/3 SCC carried somatic gene mutations. The most commonly affected gene was the *PIK3CA* gene in hot spot regions E545K and E453K in 5/8 (62%) pT1a SCC, 7/15 (70%) pT1b SCC and 10/15 (66%) pT2/3 SCC followed by *FBXW7* ($n = 4$), *KRAS* and *RB1* ($n = 2$ each). *ERBB2, APC, ATM, MLP* gene mutations occurred only once. Solitary activating oncogenic somatic mutations dominated over tumor suppressor mutation in 88% pT1a, 80% pT1b and 60% pT2/3 SCC. Concomitant mutations involved typically an activating oncogenic mutation and an inactivating tumor-suppressor gene mutation. All patients with pT1a SCC are alive without evidence of disease after surgical treatment, independent of mutational status or lympho-vascular space invasion.

Conclusions. Activating oncogenic gene mutations, in particular in the *PIK3CA* gene, occur early in cervical carcinogenesis. Although driver gene mutations bestow tumor cells with a growth advantage, early detection and complete removal of all cancer cells – with or without somatic gene mutations – are essential for cure. In contrast to advanced inoperable SCC, where *PIK3CA* driver gene mutations carry an adverse prognosis, the mutational status in surgically treated micro-invasive SCC is prognostically irrelevant.

List of abbreviations

MAF	Mutational allele frequency
HPV	human papilloma virus
HSIL	high grade squamous intraepithelial lesion
SCC	squamous cell carcinoma

1. Introduction

Despite a large body of literature, our knowledge about the temporal sequence in acquisition of genetic aberrations during cervical carcinogenesis, particularly in early invasive cervical squamous cell carcinomas (SCC) is scant and incomplete. Invasive SCC arise after persistent and transforming infections with HPV-high-risk genotypes and to a lesser degree with potentially carcinogenic HPV-subtypes [1–3]. Cervical precursor lesions

* Corresponding author.
E-mail address: sigrid.regauer@medunigraz.at (S. Regauer).

https://doi.org/10.1016/j.ygyno.2022.01.020
0090-8258/

high-grade squamous intraepithelial lesion (HSIL) develop through action of HPV oncogenes E6 and E7 [4] independent of somatic gene mutations in the hot spot region of the 50 most common cancer genes [5,6]. In contrast, numerous gene mutations are identified in invasive cervical cancers [7–12]. Driver mutations in the *PIK3CA* gene are most commonly identified and linked to an unfavorable prognosis. These study cohorts, however, varied greatly with respect to size and tumor stage. The number of investigated SCC ranged from as few as 5 SCC total [8], over 25 SCC [9], 36 SCC [12], 40 SCC [7], 145 SCC [10] to as many as 300 SCC [11], and focused mostly on advanced inoperable SCC. Some studies gave no information about tumor stages of analyzed tumors [9,12]. Early invasive pT1a SCC were the exception with 1 or 2 cases [7,8], and other studies excluded pT1a SCC altogether from analysis [9,11,12]. These studies provided no information about the temporal sequence of genetic events in the progression of micro-invasive to frankly invasive SCC. To gain better insights in the sequential steps in cervical carcinogenesis and the prevalence of mutations in different tumor stages, we analyzed a series of 32 pT1a SCC in comparison to 28 pT1b SCC, and 27 advanced pT2/3 SCC. Special focus was placed on driver gene mutation represented in cancer hot spot panel of 50 cancer genes, in particular the *PIK3CA* gene.

2. Material and methods

32 micro-invasive pT1a SCC and 55 frankly invasive SCC (28pT1b, 22 pT2, 5pT3) were identified in the archives of the Diagnostic- and Research Institute of Pathology, Medical University Graz. A control group included 85 thin and tick HSIL [6].

2.1. Mutational analysis

NGS libraries for mutational screening were prepared using the AmpliSeq library kit 2.0 (Thermo Fisher Scientific) and the Ion Ampliseq Cancer Hotspot Panel V2 (Cat Nr: 4475346) primer pool covering hot spot mutations in 50 genes implicated in cancer. Sequencing was done on an Ion GenestudiobS5XL benchtop sequencer (Thermo Fisher Scientific) to a length of 200 base pairs and initial data were analyzed using the Ion Torrent Suite Software Plug-ins (Thermo Fisher Scientific, open source, GPL, https://github.com/iontorrent/). Briefly, this included base calling, alignment to the reference genome (HG19) using the TMAP mapper and variant calling by a modified diBayes approach taking into account the flow space information. The following genes were analyzed: *ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, EZH2, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53, VHL*. Proper PCR Amplification of all amplicons and even distribution of NGS reads was documented in coverage analysis and plots of each sample. Called variants were annotated using open source software ANNOVA [13] and SnpEff [14]. All coding, nonsynonymous mutations were further evaluated and visually inspected in IGV (http://www.broadinstitute.org/igv/) and variant calls resulting from technical read errors or sequence effects were excluded from the analysis.

Chromosomal copy number variations were determined by low density whole genome sequencing. Library preparation was performed using the NEBNext® Fast DNA Library Prep Set for Ion Torrent (New England Biolabs) from 50 ng DNA according to manufactureres recommendations, and sequencing was done on Ion Torrent S5XL to a depth of approximately 5 million reads per sample. Sequence data was aligned to the reference genome (hg19) and copy number variations were called using the bioconductor package CNAnorm [15]. IRB approval was obtained (31–049 ex 18/19).

3. Results

3.1. Micro-invasive pT1a SCC

Median age of the 32 patients was 39 years (range 23–58 years; 28 pT1a1 and 4 pT1a2 SCC; 4/32 with lympho-vascular space invasion (LVSI)). All but one SCC were induced by HPV-high risk (17 HPV16; 3 HPV16 & others; 3 HPV18; 2 HPV 33; 2 "other" HR-HPV; one each HPV31; HPV58; HPV16 and 18; HPV16 and 59; details see Table 1). In one SCC possibly carcinogenic HPV73 subtype was identified. SCCs with a single focus too small for microdissection (Fig. 1a / b, total of 10) were not included in this study. Invasive SCC of about 1 mm or greater in any dimension in the 32 SCC were subjected to analysis (Fig. 1c–h, Fig. 2a–h). All patients had an extensive thick HSIL >10 cell layers thick. Early invasive cancer corresponded either to a keratinized SCC (Fig. 1a–e, 2b and c), sharply contrasting the basaloid differentiation of the HSIL from which they originated, or showed basaloid differentiation with only rudimentary keratinization. Three pT1a SCC had a syncytial-like invasion pattern (Fig, 2 g). Occasionally, the dense inflammatory infiltrate made unequivocal recognition of invasion difficult in Hematoxylin and Eosin-stained sections (Fig. 1d-f, 2f and 2h). The invasive foci were delineated by an edematous stromal reaction and/or an partly dense inflammatory response. Most SCC revealed a dense lymphocytic infiltrate (Fig. 1c–g; 2a,c–h), but invasion was easily recognized on $p16^{ink4a}$ stains (Fig. 2c and d). LVSI was present in 4/32 SCC (three pT1a2 SCC and one pT1a SCC). Treatment was cone excision ($n = 23$), sentinel lymph node dissection ($n = 3$) or simple hysterectomy ($n = 9$). All patients are presently alive without evidence of disease after a follow up of 24 months (range 6 to 107 months), including the four patients with LVSI (with 12, 18, 24 and 53 months follow-up).

In the micro-dissected samples of the micro-invasive foci, mutations in the hot spot regions of cancer genes were detected in 8/32 (25%) SCC. These included activating mutations in the *PIK3CA* gene in 5/8 (62%) SCC in the hot spot regions E545K ($n = 4$; Fig. 2b–d) and E453K ($n = 1$; Fig. 2a), and in the *ERBB* (Her-2 neu) gene in 1/32 SCC (3%; Fig. 2e–f). Two of 32 SCC (6%) had likely pathogenic somatic mutations in the tumor suppressor gene *FBXW7*. One of these SCC harbored *FBXW7* D399N (n = 1; Fig. 2g–h), and the other SCC displayed *FBXW7* R505G with a concomitant *TP53* gene mutation S241F (n = 1; likely pathogenic). The micro-dissected areas of micro-invasion contained numerous non-neoplastic inflammatory cells and portions of HSIL. Therefore, the content / percentage of analyzed cancer cells was quite low, typically between 20 and 40%. Accordingly, the MAF of somatic mutations was quite low and ranged between 4% and 21%, typically around 10%. In 9/29 (31%) SCC with sufficient DNA, a gain of chromosome arm 3q was identified, four of which occurred together with a somatic gene mutation.

3.2. Frankly invasive SCC ≥ pT1b

This group included 28 pT1b SCC with and without lymph vessel invasion / lymph node metastasis, 22 pT2 SCC, 5 pT3 SCC (see Tables 2 and 3). Median age of patients with pT1b SCC was 44 years (range 22 to 69 years), that of patients with pT2 and pT3 SCC 53 years (range 38 to 72 years). Overall, 34/55 patients (62%) had regional lymph node metastases at primary clinical presentation, and 14/55 (25%) patients died of disease after average 15 months (range 5 to 30 months) after initial diagnosis. Somatic gene mutations were identified in 26/55 (47%) invasive SCC. Overall, 18 of the mutated 26 (69%) SCC showed *PIK3CA* driver gene mutations in hot spot regions E545K and E453K. Only three SCC had a concomitant activating *PIK3CA* gene and an inactivating tumor suppressor gene mutation (*FBWX7* and *ATM* resp.).

3.2.1. pT1b SCC

All 28 pT1b SCC were induced by HPV-high risk (18 HPV16; 2 HPV45; 2 HPV58; one each HPV18, HPV33, HPV31, HPV59, HPV39,

Table 1
Genetic aberrations and CNV in invasive cervical SCC pT1a.

Patients	Age at Dx	TNM	Lympho-vascular space invasion	HPV subtypes	Somatic gene mutations Cancer Hot Spot Panel (50 genes)		Copy number variation gain of 3q	Figures
#1	23	pT1a1		HR-16	**ERBB2**	exon 20; V777L	insuff. DNA.	2e,f
#2	25	pT1a		HR-16			gain 3q	
#3	27	pT1a2	L1	HR-31			gain 3q	
#4	28	pT1a		HR-16			none	
#5	29	pT1a1		"other" HR			none	
#6	29	pT1a1		HR-16	**PIK3CA**	exon10; E545K	none	2c,d
#7	31	pT1a2	L1	16 & "other	**PIK3CA**	exon 8; E453K	none	2a
#8	33	pT1a		HR-16			gain 3q	1e
#9	33	pT1a1		possibly carcinogenic HPV 73			none	1 g
#10	34	pT1a1		HR 16 & HR-59			none	
#11	34	pT1a1		HR-33			none	
#12	35	pT1a1(m)		HR-16			gain 3q	
#13	35	pT1a1		HR-18			none	
#14	35	pT1a1		HR 16 & HR-18			insuff. DNA.	1b
#15	35	pT1a1		HR-16			none	1c
#16	36	pT1a		HR-18			gain 3q	
#17	37	pT1a1		HR-16			none	
#18	37	pT1a1		HR-16			gain 3q	1d
#19	38	pT1a1		HR 16 & "other"	**FBXW7**	exon 8; D399N	gain 3q	2 g,h
#20	38	pT1a		"other" HR			none	
#21	38	pT1a1		HR-58			none	
#22	39	pT1a1		HR-33	**PIK3CA**	exon10; E545K	insuff. DNA.	
#23	39	pT1a2	L1	HR-16			3q	
#24	40	pT1a1		HR-16	**PIK3CA**	exon10; E545K	insuff. DNA.	2b
#25	42	pT1a1		HR-16			none	
#26	43	pT1a2		16 & "other"	**PIK3CA**	exon10; E545K	gain 3q	
#27	45	pT1a		HR-18			none	1 h
#28	48	pT1a1(m)		HR-16			none	
#29	54	pT1a1	L1	HR-16			loss 3q	
#30	67	pT1a1		HR-16			none	
#31	68	pT1a1		HR-16			none	
#32	88	pT1a1		HR-16	**FBXW7** **TP53**	exon10; R505G exon 6; S241F	gain 3q	

HPV "others"). Surgical removal and histological analysis of iliac sentinel lymph nodes identified metastases in 10/28 SCC (36%). 18/28 pT1b SCC were classified as localized tumors without lymph node metastases. One of these 18 SCC, however, showed LVSI in the primary SCC. Overall, 10/28 (36%) pT1b SCC had somatic mutations, seven of 10 (70%) involved the *PIK3CA* gene at E545K ($n = 5$) and E453K ($n = 2$). All but one *PIK3CA* driver gene mutations were solitary events. One each of 28 SCC featured activating gene mutations in the *KRAS* gene (concomitant *KRAS* A146V and A146T mutations on different alleles) and a deletion/insertion in the tumor suppressor gene *FBXW7*; details see Table 2). When stratified according to LVSI or lymphatic metastasis at primary diagnosis, 6/11 (55%) SCC had mutations in the PIK3CA gene ($n = 4$), the KRAS gene ($n = 1$), and a deletion/insertion in the *FBXW7* gene (n = 1). Four of 17 (24%) pT1b SCC without LVSI had mutations (3× *PIK3CA* gene and 1× *FBXW7*). All 5/28 (18%) women, who died of disease 16 months after diagnosis (range 14 to 21 months), had lymph node metastases / LVSI, but no mutations in genes evaluated by the hot spot cancer panel.

3.2.2. pT2 and pT3 SCC

26/27 of advanced SCC were induced by HPV-high risk (18 HPV16; 2 HPV45; one each HPV16 & 52; HPV18; HPV32). Two SCC were induced by "other" HR-HPV; 2 SCC were induced by possibly carcinogenic HPV67 and HPV73 (details see Table 3). All but one patients had lymph node metastases or LVSI. 15/27 (56%) pT2/3 SCC carried gene mutations. The *PIK3CA* gene was affected in 10/15 (66%) SCC with a single hot spot mutation at E545K ($n = 6$) and E453K ($n = 3$), and two *PIK3CA* gene mutations at R108H and L540F ($n = 1$). Activating oncogenic mutations also occurred in the *ERBB2* gene (n = 1), *MPL* gene (n = 1), and the *APC* gene (n = 1). Two SCC had a deletion of the *RB1* gene with a concomitant mutation in the *HRAS* and *KRAS* gene respectively. 9/27 (33%) patients died of disease 15 months after diagnosis (range 5 to 30 months).

4. Discussion

This is the largest study on pT1a SCC to date documenting that somatic driver gene mutations in hot spot regions of 50 cancer genes are already present in early micro-invasive tumor formations. At initial diagnosis, the prevalence of somatic gene mutations increased from 25% in pT1a SCC, to 36% in pT1b SCC and 55% in pT2/3 SCC, and dominated in metastasized SCC with 59% versus only 11% of non-metastasized SCC. The most commonly mutated gene was the *PIK3CA* gene at similar frequencies throughout the different tumor stages. The overall percentages in advanced SCC compared to those reported in the literature [10,12,16]. Activation of PIK3CA pathways, however, was not exclusively dependent on actionable gene mutations. The *PIK3CA* gene can be activated by a gain of chromosome arm 3q [17,18], as identified in about 1/3 of our pT1a SCC, or by amplifications of the *PIK3CA* gene in as many as 50% of invasive cervical SCC [16,19,20]. In addition, *PIK3CA* pathways are dysregulated in HPV-related cervical carcinogenesis already during development of HSIL, since the physiological negative PIK3CA regulation by an *intact p53* protein is abolished by binding of HPVE6 oncoprotein to p53 [21]. *ERBB2, MPL*, *APC, KRAS* and *RB1* gene mutations were sporadic events, and *FGFR3* gene mutations were absent [22,23] contrasting observations in other studies [24,25]. Overall, activating mutations vastly outnumbered tumor suppressor gene-inactivating mutations. In line with the literature on cervical SCC, the second most common mutation in our cohort occurred in the tumor suppressor gene *FBWX7* [10,26], although driver mutations were the exception in our study. Inactivating *TP53* and CDKN2A gene mutations

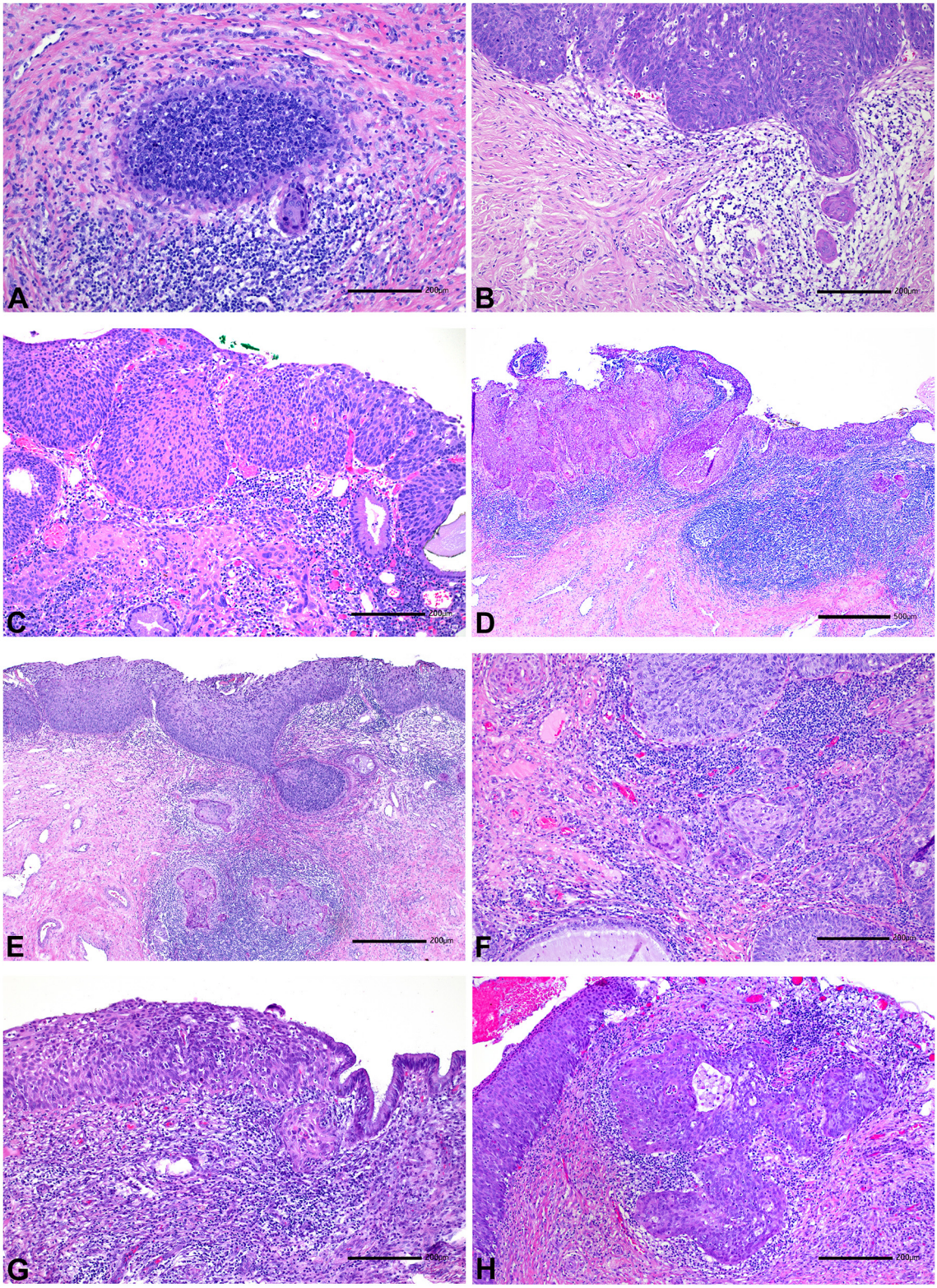

Fig. 1. pT1a SCC without somatic gene mutations.
a) An extremely early focus of beginning invasion. These minute areas could not be analyzed with the microdissection techniques and excluded from this study.
b) A basaloid thick HSIL with a microscopic focus of invasion. The peritumoral stroma is edematous with a scant lympho-histiocytic infiltrate around the invasive keratinzed tumor cells. This focus was too small for CHP analysis but other larger areas were subjected to microdissection (pt.#14).
c) A keratinizing pT1a SCC undermining a thick basaloid HSIL Neither somatic cancer hot spot gene mutations nor a gain of chromosome 3 q were identified (pt.#15).
d) A microinvasive SCC pT1a1 arising at two different sites accompanied by a dense lymhohistiocytic infiltrate. In the micro-dissected sample originating from this area, only a gain of chromosome 3q was identified (pt.#18).
e) A sharp contrast between the highly differentiated keratinizing foci of invasive SCC and the overlying thick HSIL. This specimen featured gain of chromosome 3q, but no somatic gene mutations (pt.#8).
f) A multifocally invasive pT1a SCC with invasive foci featuring basaloid differentiation with only abortive squamous differentiation. A gain of chromosome 3q was detected (pt.#12).
g) HPV73-induced early invasive SCC with micro-invasion arising adjacent to tall endocervical epithelium (pt.#9).
h) HPV18-induced SCC with micro-invasion originating from an endocervical gland involved with HSIL (pt.#27).

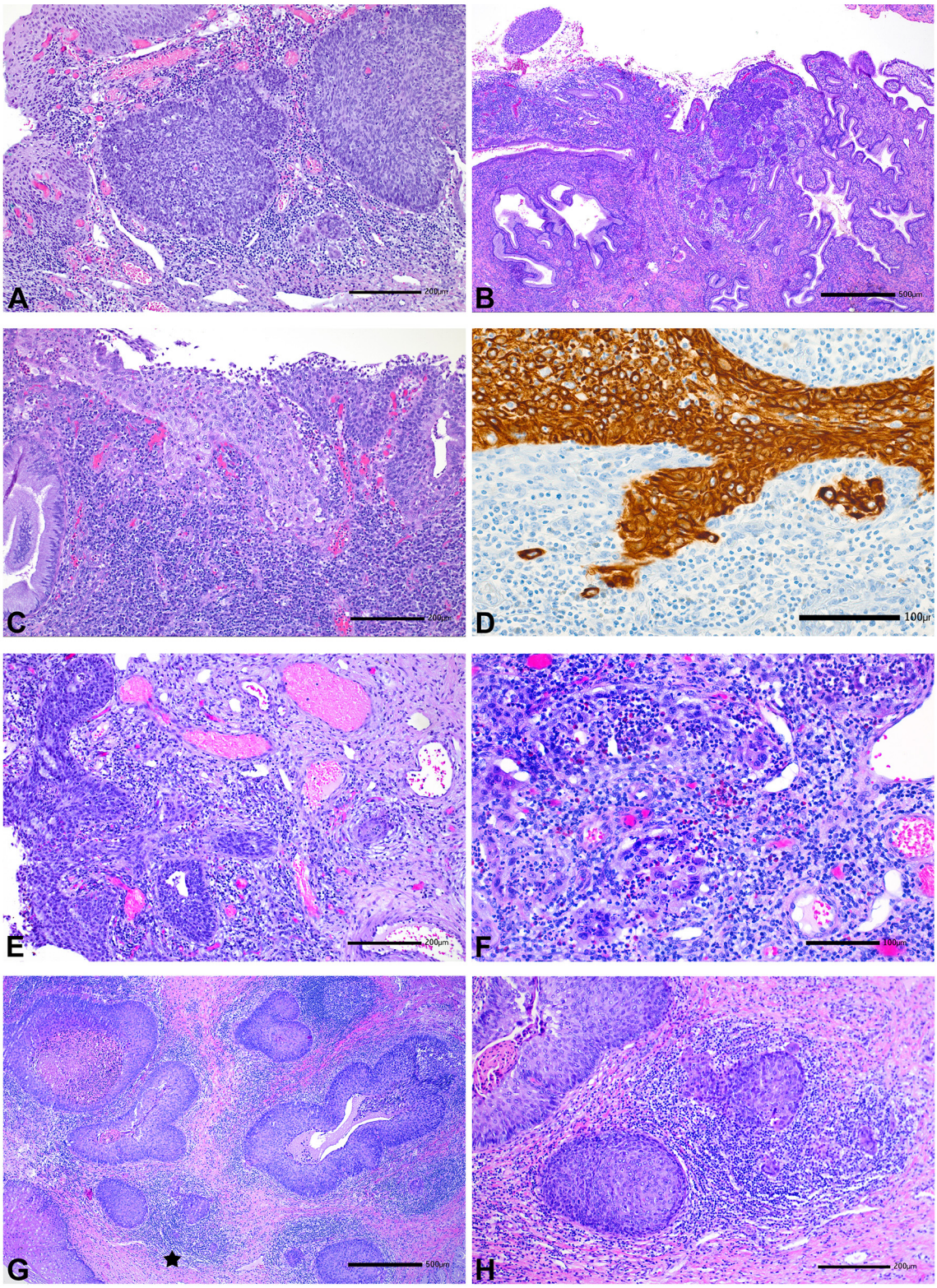

Fig. 2. pT1a SCC with somatic gene mutations.
a) A micro-invasive basaloid pT1a1 SCC with LVSI with a mutation in the PIK3CA E453K driver gene (pt.# 7).
b) A micro-invasive pT1a1 SCC featuring focal keratinization with a mutation in the PIK3CA E545K driver gene (pt.#24). Please note that the invasive SCC undermines "normal" appearing endocervical epithelium in this image. The thick HSIL present in other sections is not appreciable here.
c) Basaloid HSIL with a focus of micro-invasive SCC and an extensive dense inflammatory infiltrate. Note the change of histological differentiation in the invasive tumor component. (pt.#6).
d) p16ink4a overexpression of micro-invasive SCC illustrating the protrusion of a single tumor cell through the basement membrane and single invasive tumor cells within the dense inflammatory infiltrate (pt.#6) easily missed in the dense inflammatory infiltrate in HE stained sections.
e) A basaloid pT1a1 SCC with a ERBB2 driver mutation and a dense inflammatory infiltrate including numerous eosinophilic granulocytes (pt.#1).
f) The dense intra-tumoral and peri-tumoral inflammatory infiltrate features collections of eosinophilic granulocytes (pt.#1).
g) An extensive thick HSIL / SCC in-situ with comedo-necrosis and focal micro-invasion (asterix) featuring a mutation in the tumor suppressor gene FBXW7 (pt.#19).
h) The areas of micro-invasion are surrounded by a dense lymphocytic infiltrate (pt.#19).

Table 2
Somatic gene mutations in 28 pT1b SCC.

	Age at Dx.	HPV subtypes	1° SCC < 2 cm	1° SCC > 2 cm	1° SCC > 4 cm	reg. LN mets	CHP (50 genes) Somatic mutations		DOD (months)	Survival (months)
#1	22	HR-16			pT1b2	1	none		12	
#2	29	HR-16	pT1b			1	**PIK3CA**	exon10; E545K		
							ATM	exon8; S334L		33
#3	32	HR-16	pT1b			0				lost to F/U after 48 months
#4	33	HR-16	pT1b1			0	**PIK3CA**	exon10; E543K		80
#5	34	HR-16			pT1b2; L1; V1	1			14	
#6	34	HR-16	pT1b1			1	**PIK3CA**	exon10; E545K		22
#7	36	HR-45			pT1b2	0				24
#8	37	HR-16	pT1b			0				20
#9	37	HR-45			pT1b2	1			**17**	
#10	38	HR-16	pT1b1; L1			1	**PIK3CA**	exon10; E543K		42
#11	38	HR-16		pT1b1; L1		0				20
#12	38	HR-16	pT1b1; L1			1	**PIK3CA**	exon10; E543K		66
#13	40	HR-16			pT1b2	0				17
#14	41	HR-16		pT1b1; L1		0				6
#15	41	HR-16	pT1b1;			0				90
#16	31	HR-16	pT1b			0				144
#17	46	HR-16		pT1b, L1		0	**FBXW7**	exon 10; p.479_480delinsH		14
#18	45	HR-16			pT1b2	1			**21**	
#19	47	HR-39		pT1b		1				72
#20	48	HR-31			pT1b2	0				68
#21	49	HR-58	pT1b			0	**PIK3CA**	exon10; E545K		15
#22	51	HR-59			pT1b2; L1	0			**16**	
#23	52	HR-58	pT1b, L1			1	**K-RAS**	exon4; A146V bi-allelic		54
#24	55	HR-16	pT1b			0	**PIK3CA**	exon10; E545K		7
#25	59	HR-18			pT1b2	0				18
#26	61	HR-33	pT1b1			0				40
#27	61	HR-16	pT1b, L1			0	**PIK3CA**	exon10; E545K		68
#28	69	"other" HR		pT1b1		0				42

Table 3
Somatic gene mutations in 27 cervical SCC pT2 and pT3.

	Age at Dx.	HPV subtypes	TNM	LN mets	CHP (50 genes) Somatic mutations		Dead of disease (months)	Survival (months)
#1	38	HR-16	pT2b	1	**PIK3CA**	exon 2; R108 H		74
					PIK3CA	exon 10; L540F		
#2	40	HR-16	pT1b1	1	**MPL**	exon10; H520Q	**5**	
#3	42	HR-16		1	**none**			3
#4	43	HR-16 & HR-52	pT2b	1	**PIK3CA**	exon10; E543K		52
#5	45	HR-16	pT2b	1	**none**			4
#6	45	HR-16	pT3b	1	**none**			9
#7	45	HR-18	pT3b	1	**PIK3CA**	exon10;E545K		3
					FBXW7	exon10; R479Q		
#8	46	HR-16	pT2a	1	**PIK3CA**	exon10; E543K		8
#9	46	possibly carcinogenic HPV 73	pT2b, L1	0	**none**			26
#10	48	HR-16	pT2b	1	**ERBB2**	exon 19; I767M	**8**	
#11	48	HR-45	pT2b	1	**APC**	exon 16, A1358V		13
#12	49	HR-16	pT2a	1	**PIK3CA**	exon10;E545K	**26**	
					FBXW7	exon10; R505G		
#13	52	HR-16	pT2b	1	**PIK3CA**	exon10;E545K	**9**	
#14	53	HR-16	pT3b	1	**none**		**6**	
#15	54	HR-16	pT3b	1	**PIK3CA**	exon 8; E453K	**18**	
#16	55	HR-16	pT2b	1	**none**			110
#17	56	HR-16	pT2b	1	**none**			9
#18	56	HR-16	pT3b	1	**FBXW7**	exon9; R465H		
#19	59	HR-16	pT2b, L1	1	**PIK3CA**	exon10;E545K	**30**	
#20	59	HR-31	pT2b	0	**RB1**	exon17; W563X		3
					KRAS	exon2; G12D		
#21	61	HR-16	pT2b	1	**HRAS**	exon3; F78L		3
					RB1	exon 11; R358X		
#22	61	HR-16	pT2a2, L1	1	**PIK3CA**	exon10;E545K		62
#23	65	HR-16	pT2b	1	**none**			114
#24	68	possibly carcinogenic HPV 67	pT2b	1	**PIK3CA**	exon10; E545K	**27**	
#25	69	"other" HR	pT2b	1	**none**			3
#26	71	"other" HR	pT2b	1	**none**			17
#27	72	HR-45	pT2b	0	**none**		8	

were absent in our HPV-driven SCC, but *TP53* gene mutations in cervical SCC display geographical differences [27] and in cell lines are most likely the result of continued passage [28].

Driver gene mutations were present in early stage cervical SCC, with *PIK3CA* gene mutations dominating as described previously for advanced late tumor stages. The relatively low prevalence of 25% somatic gene mutations in pT1a SCC needs careful interpretation as technical limitations of the microdissection technique in detecting individual cells with a point mutation may account for false negative results. DNA of HSIL may have dominated over that of the small invasive foci, and/or the numerous non-neoplastic inflammatory cells in the micro-dissected samples may have "diluted" signals from a small clone with a mutated base below detection level. Limitations of this technique were demonstrated in a comparative study of primary pancreatic cancer with its cell line, when only 63% of driver mutations detected in the cancer cell enriched cell line could be verified in the micro-dissected tumor specimen [29,30]. These technical aspects may have led to exclusion of early invasive SCC from previous analyses [7–12]. The emergence of gene mutations in pT1a SCC coincides with invasion, as somatic mutations are absent from cervical precursor lesions [5,6]. The process of invasion through the basement membrane is poorly understood. Aside from degradation of the basement membrane by upregulation of proteinases, physical forces such as the pure pressure of the bulk of cells comprising the thick HSIL, specifically of dividing cells, may push cells through physiological gaps in the basement membrane [31,32]. In addition, lymphocyte infiltrates or cytokine secretion of tumor infiltrating lymphocytes further weaken the basement membrane. It is unclear at present, if driver gene mutations in individual tumor cells (not detectable by present techniques) are necessary for basement membrane penetration, or if they are acquired immediately after initial invasion, and if they contribute to a change in the differentiation program in early invasive tumor cells. The gradual increase of *PIK3CA* driver gene mutations from pT1 to pT3 SCC suggest, however, that some mutations are acquired in later tumor stages.

Activated PIK3CA pathways and/or *PIK3CA* driver gene mutations acquired early around the time of invasion provide initially only a minimal selective growth advantage for each individual cell. It takes numerous cell divisions before a selective growth advantage conferred by driver mutations establishes a large cancer that has escaped to surgically inaccessible sites. Driver gene mutations affecting different pathways such as cell survival (e.g. *PIK3CA*, growth factor receptors), genome maintenance (*TP53, ATM, FBXW7, CDKN2A*) or cell fate (*APC*) [33] impact not only on tumor progression but on therapy decisions. Mutation specific therapy is available mostly for oncogenic activating mutations as these drugs interfere with protein functions. Conversely, tumor suppressor gene mutations cannot be replaced / repaired by drugs but may be targeted indirectly as is the case with synthetic lethality achieved by PARP inhibitors. The present standard first line treatment for advanced local or metastatic SCC is radio-chemotherapy. *PIK3CA* gene mutations, however, confer resistance to cisplatin-based chemotherapy and are thus linked to poor clinical outcome [34,35]. In contrast, the mutational status of adequately treated micro-invasive cervical SCC carries no prognostic value, since early detection and surgical removal of all tumor cells cures a patient.

In summary, somatic driver gene mutations are already present in micro-invasive cervical SCC. *PIK3CA* driver gene mutations vastly outnumbered other activating mutations or tumor-suppressor gene mutations. Early detection and complete removal of all cancer cells – independent of somatic gene mutations – cured the patients. Our data reinforce the concept that HSIL and adequately treated micro-invasive SCC have the same prognosis even when their genetic profile is the same as in advanced disease. Once progressed to advanced inoperable tumor stages and metastases, presence and type of driver gene mutation affects prognosis and therapy success.

Declaration of Competing Interest

All authors declare no conflict of interest.

References

[1] H. Zur Hausen, Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis, J. Natl. Cancer Inst. 92 (2000) 690–698.
[2] H. Zur Hausen, Papillomaviruses and cancer: from basic studies to clinical application, Nat. Rev. Cancer 2 (2002) 342–350.
[3] G. Halec, L. Alemany, B. Lloveras, M. Schmitt, M. Alejo, F.X. Bosch, S. Tous, J.E. Klaustermeier, N. Guimera, N. Grabe, et al., Pathogenic role of the eight probably/possibly carcinogenic HPV types 26, 53, 66, 67, 68, 70, 73 and 82 in cervical cancer, J. Pathol. 234 (2014) 441–451.
[4] S. Regauer, O. Reich, K. Kashofer, Thin variant of high-grade squamous intraepithelial lesion - relationship with high-risk and possibly carcinogenic human papilloma virus subtypes and somatic cancer gene mutations, Histopathology 75 (2019) 405–412.
[5] W. Verlaat, P.J. Snijders, M.I. van Moorsel, M. Bleeker, L. Rozendaal, D. Sie, B. Ylstra, C.J. Meijer, R.D. Steenbergen, D.A. Heideman, Somatic mutation in PIK3CA is a late event in cervical carcinogenesis, J. Pathol. Clin. Res. 1 (2015) 207–211.
[6] S. Regauer, O. Reich, K. Kashofer, Cervical Precancers originate from infected proliferating reserve cells: a comparative histologic and genetic study of thin and thick high-grade squamous intraepithelial lesions, Am. J. Surg. Pathol. (2021) https://doi.org/10.1097/PAS.0000000000001818.
[7] A.A. Wright, B.E. Howitt, A.P. Myers, S.E. Dahlberg, E. Palescandolo, P. Van Hummelen, L.E. MacConaill, M. Shoni, N. Wagle, R.T. Jones, et al., Oncogenic mutations in cervical cancer: genomic differences between adenocarcinomas and squamous cell carcinomas of the cervix, Cancer 119 (2013) 3776–3783.
[8] S.H. Jung, Y.J. Choi, M.S. Kim, I.P. Baek, S.H. Lee, A.W. Lee, S.Y. Hur, T.M. Kim, S.H. Lee, Y.J. Chung, Progression of naive intraepithelial neoplasia genome to aggressive squamous cell carcinoma genome of uterine cervix, Oncotarget 6 (2015) 4385–4393.
[9] E. Muller, B. Brault, A. Holmes, A. Legros, E. Jeannot, M. Campitelli, A. Rousselin, N. Goardon, T. Frebourg, S. Krieger, et al., Genetic profiles of cervical tumors by high-throughput sequencing for personalized medical care, Cancer Med. 4 (2015) 1484–1493.
[10] Cancer Genome Atlas Research N, Albert Einstein College of M, Analytical Biological S, Barretos Cancer H, Baylor College of M, Beckman Research Institute of City of H, Buck Institute for Research on A, Canada's Michael Smith Genome Sciences C, Harvard Medical S, Helen FGCC, et al., Integrated genomic and molecular characterization of cervical cancer, Nature 543 (2017) 378–384.
[11] S. Scholl, M. Popovic, A. de la Rochefordiere, E. Girard, S. Dureau, A. Mandic, K. Koprivsek, N. Samet, M. Craina, M. Margan, et al., Clinical and genetic landscape of treatment naive cervical cancer: alterations in PIK3CA and in epigenetic modulators associated with sub-optimal outcome, EBioMedicine 43 (2019) 253–260.
[12] T. Watanabe, H. Nanamiya, M. Kojima, S. Nomura, S. Furukawa, S. Soeda, D. Tanaka, T. Isogai, J.I. Imai, S. Watanabe, et al., Clinical implication of oncogenic somatic mutations in early-stage cervical cancer with radical hysterectomy, Sci. Rep. 10 (2020) 18734.
[13] K. Wang, M. Li, H. Hakonarson, ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data, Nucleic Acids Res. 38 (2010), e164.
[14] P. Cingolani, A. Platts, L. Wang, M. Coon, T. Nguyen, L. Wang, S.J. Land, X. Lu, D.M. Ruden, A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3, Fly (Austin) 6 (2012) 80–92.
[15] A. Gusnanto, H.M. Wood, Y. Pawitan, P. Rabbitts, S. Berri, Correcting for cancer genome size and tumour cell content enables better estimation of copy number alterations from next-generation sequence data, Bioinformatics 28 (2012) 40–47.
[16] J.B. McIntyre, J.S. Wu, P.S. Craighead, T. Phan, M. Kobel, S.P. Lees-Miller, P. Ghatage, A.M. Magliocco, C.M. Doll, PIK3CA mutational status and overall survival in patients with cervical cancer treated with radical chemoradiotherapy, Gynecol. Oncol. 128 (2013) 409–414.
[17] P.H. Rao, H. Arias-Pulido, X.Y. Lu, C.P. Harris, H. Vargas, F.F. Zhang, G. Narayan, A. Schneider, M.B. Terry, V.V. Murty, Chromosomal amplifications, 3q gain and deletions of 2q33-q37 are the frequent genetic changes in cervical carcinoma, BMC Cancer 4 (2004) 5.
[18] W. Verlaat, P.J.F. Snijders, P.W. Novianti, S.M. Wilting, L.M.A. De Strooper, G. Trooskens, J. Vandersmissen, W. Van Criekinge, G.B.A. Wisman, C. Meijer, et al., Genome-wide DNA methylation profiling reveals methylation markers associated with 3q gain for detection of cervical Precancer and Cancer, Clin. Cancer Res. 23 (2017) 3813–3822.
[19] Y.Y. Ma, S.J. Wei, Y.C. Lin, J.C. Lung, T.C. Chang, J. Whang-Peng, J.M. Liu, D.M. Yang, W.K. Yang, C.Y. Shen, PIK3CA as an oncogene in cervical cancer, Oncogene 19 (2000) 2739–2744.
[20] K. Martell, J.B. McIntyre, E.N. Kornaga, A.M.Y. Chan, T. Phan, M. Kobel, E.K. Enwere, M.L. Dean, P. Ghatage, S.P. Lees-Miller, et al., PIK3CA mutation and CNV status and post-chemoradiotherapy survival in patients with cervical cancer, Gynecol. Oncol. 158 (3) (2020) 776–784.
[21] B. Singh, P.G. Reddy, A. Goberdhan, C. Walsh, S. Dao, I. Ngai, T.C. Chou, P OC, A.J. Levine, P.H. Rao, et al., p53 regulates cell survival by inhibiting PIK3CA in squamous cell carcinomas, Genes Dev. 16 (2002) 984–993.
[22] R. Wu, D. Connolly, C. Ngelangel, F.X. Bosch, N. Munoz, K.R. Cho, Somatic mutations of fibroblast growth factor receptor 3 (FGFR3) are uncommon in carcinomas of the uterine cervix, Oncogene 19 (2000) 5543–5546.

[23] C.J. Yee, O. Lin, J. Boyd, Analysis of fibroblast growth factor receptor 3 S249C mutation in cervical carcinoma, J. Natl. Cancer Inst. 92 (2000) 1848–1849.
[24] D. Cappellen, C. De Oliveira, D. Ricol, S. de Medina, J. Bourdin, X. Sastre-Garau, D. Chopin, J.P. Thiery, F. Radvanyi, Frequent activating mutations of FGFR3 in human bladder and cervix carcinomas, Nat. Genet. 23 (1999) 18–20.
[25] C. Rosty, M.H. Aubriot, D. Cappellen, J. Bourdin, I. Cartier, J.P. Thiery, X. Sastre-Garau, F. Radvanyi, Clinical and biological characteristics of cervical neoplasias with FGFR3 mutation, Mol. Cancer 4 (2005) 15.
[26] F. Liu, Y. Zou, F. Wang, B. Yang, Z. Zhang, Y. Luo, M. Liang, J. Zhou, O. Huang, FBXW7 mutations promote cell proliferation, migration, and invasion in cervical Cancer, Genet. Test Mol. Biomark. 23 (2019) 409–417.
[27] M.L. Tornesello, L. Buonaguro, F.M. Buonaguro, Mutations of the TP53 gene in adenocarcinoma and squamous cell carcinoma of the cervix: a systematic review, Gynecol. Oncol. 128 (2013) 442–448.
[28] B. Leroy, L. Girard, A. Hollestelle, J.D. Minna, A.F. Gazdar, T. Soussi, Analysis of TP53 mutation status in human cancer cell lines: a reassessment, Hum. Mutat. 35 (2014) 756–765.
[29] S. Jones, X. Zhang, D.W. Parsons, J.C. Lin, R.J. Leary, P. Angenendt, P. Mankoo, H. Carter, H. Kamiyama, A. Jimeno, et al., Core signaling pathways in human pancreatic cancers revealed by global genomic analyses, Science 321 (2008) 1801–1806.
[30] A.V. Biankin, N. Waddell, K.S. Kassahn, M.C. Gingras, L.B. Muthuswamy, A.L. Johns, D.K. Miller, P.J. Wilson, A.M. Patch, J. Wu, et al., Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes, Nature 491 (2012) 399–405.
[31] J.A. Engbring, H.K. Kleinman, The basement membrane matrix in malignancy, J. Pathol. 200 (2003) 465–470.
[32] J. Chang, O. Chaudhuri, Beyond proteases: basement membrane mechanics and cancer invasion, J. Cell Biol. 218 (2019) 2456–2469.
[33] B. Vogelstein, N. Papadopoulos, V.E. Velculescu, S. Zhou, L.A. Diaz Jr., K.W. Kinzler, Cancer genome landscapes, Science 339 (2013) 1546–1558.
[34] A. de la Rochefordiere, M. Kamal, A. Floquet, L. Thomas, P. Petrow, T. Petit, M. Pop, M. Fabbro, C. Kerr, F. Joly, et al., PIK3CA pathway mutations predictive of poor response following standard Radiochemotherapy +/− Cetuximab in cervical Cancer patients, Clin. Cancer Res. 21 (2015) 2530–2537.
[35] B. Lachkar, T. Minaguchi, A. Akiyama, S. Liu, S. Zhang, C. Xu, A. Shikama, N. Tasaka, M. Sakurai, S. Nakao, et al., Prognostic significance of PIK3CA mutation in stage IIB to IVA cervical cancers treated by concurrent chemoradiotherapy with weekly cisplatin, Medicine (Baltimore) 97 (2018), e11392.