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Molecular Correlates of Invasion Pattern in HPV-Associated Endocervical Adenocarcinoma: Emergence of Two Distinct Risk-Stratified Tiers

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Abstract

Introduction: The pattern-based (Silva) classification of invasive HPV-associated endocervical adenocarcinomas (HPVA) is an established and reproducible method to predict outcomes for this otherwise stage-dependent group of tumors. Previous studies utilizing targeted sequencing have shown a correlation between mutational profiles and invasive pattern. However, such correlation has not been explored using comprehensive molecular testing.

Design: Clinicopathologic data including invasive pattern (Silva groups A, B, and C) was collected for a cohort of invasive HPVA, which previously underwent massive parallel sequencing using a panel covering 447 genes. Pathogenic alterations, molecular signatures, tumor mutational burden (TMB), and copy number alterations (CNA) were correlated with pattern of invasion.

Results: 45 HPVA (11 pattern A, 17 pattern B and 17 pattern C tumors) were included. Patients with pattern A presented at stage I with no involved lymph nodes or evidence of recurrence (in those with > 2 months of follow-up). Patterns B and C patients also mostly presented at stage I with negative lymph nodes but had greater frequency of recurrence. 3/17 pattern B and 1/17 pattern C HPVAs harbored lymphovascular space invasion (LVI). APOBEC mutational signature was detected only in Silva pattern C tumors (5/17), and pathogenic *PIK3CA*

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Author Contributions

AES and CPH performed study concept, design, statistical analysis, and manuscript writing. CPH, AJH, KJP, MRN, BEH, EOM, and BD provided acquisition, analysis, and interpretation of data. All authors read and approved the final paper.

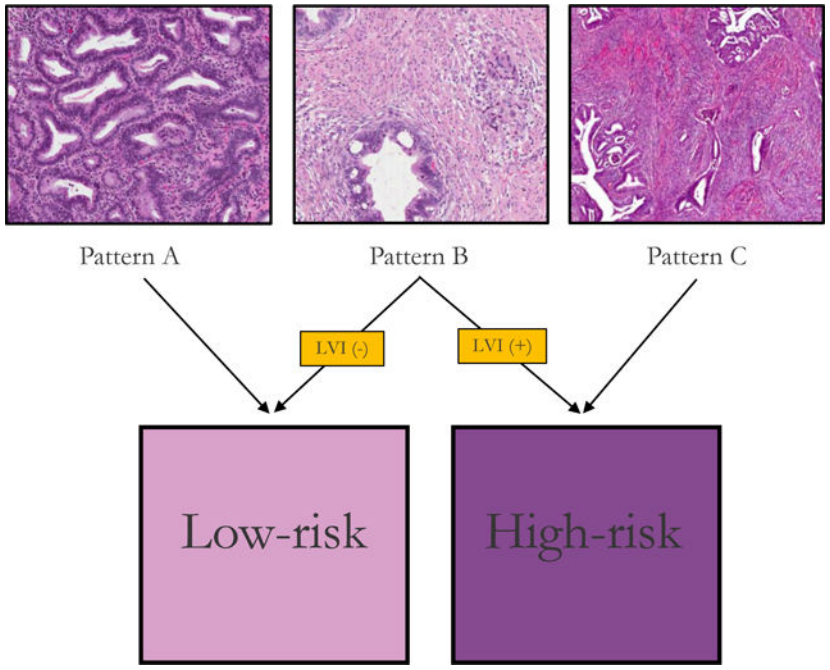
Conflicts of Interest

The authors have disclosed that they have no significant relationships with, or financial interest in, any commercial companies pertaining to this article.

changes were detected only in destructively invasive HPVA (patterns B and C). When cases were grouped as low-risk (pattern A and pattern B without LVI) and high-risk (pattern B with LVI and pattern C), high-risk tumors were enriched in mutations in *PIK3CA*, *ATRX*, and *ERBB2*. There was a statistically significant difference in TMB between low-risk and high-risk pattern tumors (p=0.006), as well as between Pattern C tumors with and without an APOBEC signature (p=0.002). CNA burden increased from pattern A to C.

Conclusions: Our findings further indicate that key molecular events in HPVA correlate with the morphologic invasive properties of the tumor and their aggressiveness. Pattern B tumors with LVI clustered with pattern C tumors, whereas pattern B tumors without LVI approached pattern A genotypically. Our study provides a biologic foundation for consolidating the Silva system into low-risk (pattern A + B without LVI) and high-risk (pattern B with LVI and pattern C) categories.

Abstract



This study compares the genomic landscapes between the three pattern-based (Silva) tiers of HPV-associated endocervical adenocarcinoma, illustrating a possible association between destructive invasion and degree of genomic injury. It provides molecular corroboration for a simplified, 2-tiered low-risk and high-risk pattern-based system.

Keywords

invasive endocervical adenocarcinoma; HPV; Silva classification; Next generation sequencing; APOBEC; *ERBB2* ; *PIK3CA*

Introduction

Risk stratification of endocervical adenocarcinoma is a challenging task, and our understanding of the pathophysiology, histology, and outcome of these diverse tumors has evolved significantly in the past decade. In addition to the paradigm shift of HPV-based histologic subtyping,[1–2] the pattern-based Silva classification of invasive HPV-associated endoervical adenocarcinoma (HPVA) is increasingly recognized as a reproducible and accurate method for extrapolating tumor behavior.[3–6] However, the current FIGO staging system for cervical cancer relies largely on problematic and inconsistent measurements of tumor size and depth of invasion, especially in early-stage disease. In turn, as these parameters influence clinical decision-making with regards to lymph node assessment, a subset of low-risk patients are subject to morbidity from invasive adjunct procedures.[7]

The 2013 three-tiered Silva system is predicated primarily on the presence or absence of destructive stromal invasion. As evident in multiple independent institutional cohorts, this system shows not only external validity but also inter-observer reproducibility (compared to FIGO stage and superseding other grading/classification systems for reliably predicting outcomes[8–10]) which have merited its incorporation into the upcoming National Comprehensive Cancer Network Guidelines® for cervical cancer.[7] Nonetheless, further study including prospective clinical trials is required before it is universally sanctioned and acted upon in daily practice. In this regard, it has been recently shown that, based on patient outcome, patients can be subdivided in two groups: low-risk (those with pattern A and pattern B without lymphovascular invasion [LVI]) and high-risk (pattern C and pattern B with LVI) with simpler and superior stratification compared to the original three-tiered system.[42]

A few studies have illustrated the contrasting genomic landscape of HPV-associated endocervical adenocarcinomas stratified by pattern. These studies have shown that destructively invasive tumors (patterns B and C) have a significantly higher number of single nucleotide variations (SNV) in several protooncogenes and tumor suppressor genes, when compared to pattern A lesions. However, this prior evidence has been gathered on small cohorts using limited gene panels.[11, 15] Herein, we sought to correlate each invasive pattern, both as originally defined and subdivided in the recently proposed low-risk and high-risk categories, with the underlying molecular tumor characteristics using comprehensive next generation sequencing, in an effort to determine if there is indeed an underlying biologic stratification commensurate with either system.

Materials

This study was approved by the Institutional Review Boards at Sunnybrook Health Sciences Centre (Toronto, ON, Canada) and Brigham and Women's Hospital (Boston, MA)

Cohort Selection

Cases of invasive HPVA were identified from an initial cohort of endocervical adenocarcinomas as previously described[12], spanning the years 2002–2018. HPV-positive status was confirmed via HPV *in situ* hybridization in all cases as previously reported.[12]

Cases with HPV-independent status, or mixed morphology endocervical adenocarcinoma (ie, those with a 'usual-type' component admixed with neuroendocrine and/or squamous elements) were excluded. Cases lacking formalin-fixed, paraffin-embedded (FFPE) tissue blocks, complete slide sets of the entirely submitted tumor, or clinical data were also excluded.

Clinicopathologic Review

The clinicopathologic data, including age and FIGO stage at diagnosis, type of resection (cone/loop electrosurgical excision procedure (LEEP)/trachelectomy/hysterectomy), depth of invasion, horizontal spread, margin status, presence of LVI, and lymph node status, was previously collected[12] , and for the purposes of this study retrieved in order to analyze based on pattern of invasion. Follow-up information, also formerly collected, included presence of, time to, and site of recurrence, along with time to last follow-up.

Evaluation of invasive pattern was performed on cone/LEEP/trachelectomy/hysterectomy specimens.[6] Pattern of invasion was assigned by two pathologists (AJH, CPH), working cooperatively at a multiheaded microscope, and blinded to stage or lymph node status. All tumor-containing slides were evaluated. Strict criteria for assignation of pattern A, B, or C to each case was performed using the WHO-endorsed criteria for pattern-based classification.[34, 37]

DNA Extraction and Sequencing

Next Generation Sequencing was performed on select FFPE tissue blocks at the Center for Advanced Molecular Diagnostics at Brigham and Women's Hospital (Boston, MA). After pathologist assessment of tumor cellularity (cutoff >20%), DNA was isolated from 4μm FFPE sections using a commercially available kit from Qiagen (Valencia, CA). After DNA quantification with PicoGreen, samples with 50ng/μL or greater proceeded to hybrid capture library preparation as previously described.[12] Paired normal tissue samples were not concurrently analyzed.

Sheared DNA was hybridized to a set of custom-designed capture probes (Agilent SureSelect) targeting the complete exonic regions of 447 cancer genes and 191 intronic regions across 60 genes for the evaluation of structural rearrangements (see Supplemental File 1 for list of target Oncopanel genes). Sequencing was performed using Illumina HiSeq 2500.

Data were analyzed by an internally developed bioinformatics pipeline composed of reconfigured publicly available tools and internally developed algorithms (VisCap Cancer, Phaser, BreaKmer)29. Pooled sample reads were demultiplexed using Picard (http://picard.sourceforge.net/command-line-overview.shtml), aligned to Human Genome Reference Consortium reference sequence GRCh37p1331 and duplicate reads were removed. GATK32 was used to refine alignments around insertion/deletion (indel) sites. Single nucleotide variants were called using MuTect33 and indels using Indelocator (http://www.broadinstitute.org/cancer/cga/indelocator). Because tumor tissues were tested without a paired normal from individual patients, additional informatics steps were taken to identify common single nucleotide polymorphisms: any single nucleotide polymorphism present

at >0.1% in Exome Variant Server, NHLBI GO Exome Sequencing Project, Seattle, WA (URL: http://evs.gs.washington.edu.myaccess.library.utoronto.ca/EVS/) was filtered, however, variants also present in the Catalogue of Somatic Mutations in Cancer (COSMIC; cancer.sanger.ac.uk) were rescued for manual review. VisCap Cancer calls copy number changes based on log2 ratios that are calculated using a normalized depth of coverage against a median from a panel of normal (non-cancer) samples. Circular binary segmentation was used to segment the data; segments were called via strict thresholding. Unique, aligned (hg19) sequence reads with PHRED>30 were reviewed, annotated, and interpreted using Integrated Genome Viewer (Broad Institute, Cambridge, MA) and a suite of internally developed Web-based tools. Samples with a mean target coverage of <50 × were failed and excluded from further analysis. Individual variants present at <10% allele fraction or in regions with <50 × coverage were flagged for manual review and interpreted by the reviewing laboratory scientists and molecular pathologists based on overall tumor percentage, read depth, complexity of alteration, and evidence for associated copy number alterations. Manual inspection also included cross-reference with the Catalogue of Somatic Mutations in Cancer (COSMIC) database (https://cancer.sanger.ac.uk/cosmic).

In this platform, “actionable” pathogenic mutations are defined as an alteration which: a) predicts response to treatment with an FDA-approved therapy or an investigational therapy in clinical trials for the same cancer type, b) has proven association of response to treatment with an FDA-approved therapy in a different type of cancer, or c) is a similar alteration with a proven association with response with an FDA-approved therapy in this cancer type.

Statistical Analysis

For comparison between pattern groups, the unpaired t-test and ANOVA with post-hoc Bonferroni test was used. A p-value of <0.05 was considered statistically significant. Data was analyzed based on two different schemata: 1.) the conventional three-tiered pattern-based (Silva) system, and 2.) the recently proposed binary classification merging pattern A and pattern B *without* LVI HPVAs into one *‘low-risk’* tier and merging pattern B *with* LVI and pattern C HPVAs into one *‘high-risk’* tier.

Results

Clinicopathologic Features

Clinicopathologic features are summarized in Table 1. The entire cohort of HPV-associated ECA was composed of 45 patients, with ages ranging between 22 and 80 years (mean 43, median 40). Of the 42 patients for which stage could definitively be assigned, 14 presented at stage IA, 23 at stage IB, and 5 at stage II. Of the 17 patients in the cohort who underwent pelvic lymph node dissection, none had lymph node metastases at presentation. Follow-up interval ranged from 2 days to 9 years, during which 7 patients developed recurrence of disease (16%). Application of the Silva pattern criteria parsed this cohort into 11 tumors showing only morphologic evidence of pattern A, 17 of pattern B, and 17 of pattern C. Clinicopathologic information pertaining to each pattern is outlined below.

Pattern A: The 11 patients with pattern A tumors ranged in age from 22 to 80 years (mean 43, median 40). Of these, 55% presented at stage IA (6/11), 36% at stage IB (4/11), and 9% at stage II (1/11). With a median follow-up period of 42 months (range 8–102 months), none showed evidence of recurrence (0/11).

Pattern B: The 17 patients with pattern B tumors ranged in age from 23 to 62 years (mean 41, median 40). Of these, 24% presented at stage IA (4/17), 65% at stage IB (11/17), and 12% at stage II (2/17). Median follow-up was 32 months (range 2–99 months). Overall, 24% (4/17) recurred both in distant (supraclavicular lymph node, and as widespread carcinomatosis) as well as local (rectum, parametrium) sites. Of ECA with pattern B, 18% (3/17) harbored LVI, and 2 (66%) of these experienced recurrence within two years despite having negative surgical margins.

Pattern C: The 17 patients with pattern C tumors ranged in age from 26 to 78 years (mean 45, median 40). (Stage was indeterminate in 3 cases due to issues with depth of invasion and horizontal extent measurements, which did not impact assignment of Silva pattern.) Of the 14 patients with available staging data, 29% presented at stage IA (4/14), 57% at stage IB (8/14), and 6% at stage II (2/14). Median follow-up was 25 months (range 3–133 months). Overall, 12% (2/17) of tumors with pattern C recurred (one distantly to stomach, other locally to vulvar area); interestingly, these were not associated with LVI. The single tumor with LVI in this group (1/17, 6%) did not recur within the minimal available follow-up window (<1 month).

Low-risk group (pattern A + pattern B without LVI): The 25 patients with low-risk HPVA ranged in age from 22 to 80 years (mean 43, median 40). Of these patients, 40% presented at stage IA (10/25), 48% at stage IB (12/25), and 12% at stage II (3/25). As defined, none of these 25 patients demonstrate LVI. Median follow-up was 39 months. 12% (3/22) patients experienced recurrence (as above – to a supraclavicular lymph node and rectum; the third remains an unknown location).

High-risk group (pattern B with LVI + pattern C): The 20 patients with low-risk HPVA ranged in age from 23 to 78 years, (mean 44, median 40). Of the 17 patients with available staging data, 24% presented at stage IA (4/17), 65% at stage IB (11/17), and 12% at stage II (2/17). 20% (4/20) harbored LVI. Median follow-up was 27 months. 25% (4/16) recurred (as above – widespread carcinomatosis, to the stomach, and locally to the parametrium and vulva, respectively), 50% (2/4) of these were associated with LVI.

Molecular Features

Pathogenic Genomic Mutations—Recurrent SNV (ie, those identified in >2 tumors) are visualized in Figure 1, separated by ‘high-risk’ and ‘low-risk’ patterns as well as by conventional pattern of invasion. When cases were grouped as low-risk (pattern A and pattern B without LVI) and high-risk (pattern B with LVI and pattern C), high-risk tumors were enriched in deleterious SNV in *PIK3CA*, *ERBB2* and *ATRX*. *ATRX* mutations were restricted to pattern C tumors. Pathogenic *KRAS* and *GNAS* SNVs were found in

comparable frequency in all patterns, as were deleterious *TP53* mutations (identified in 5/45 tumors, 11%). Of note, all these cases had wild type p53 staining by immunohistochemistry.

Tumor Mutational Burden—A significant difference was observed in the TMB from 'low-risk' and 'high-risk' tumors (p*<0.05) on one-way ANOVA with *post-hoc* Bonferroni test. In contrast, there was no significant difference in average TMB between patterns A, B, and C as defined (p=0.14; Figure 2). None of the 45 HPVA were mismatch repair pathway deficient by Next Generation Sequencing.

APOBEC Mutational Signature—Five tumors (11%, 5/45), all of which fell into the pattern C group, had evidence of an APOBEC-mediated mutagenesis signature. Outcome was available for 3 of these 5 patients, and one patient recurred (33%). The presence of an APOBEC signature was associated with a significantly higher TMB when compared to tumors without APOBEC signature (p**<0.00001), which persisted when compared solely with the TMB of non-APOBEC pattern C HPVAs (p**<0.004).

'Actionable' Mutations—Only pattern C tumors (12%, 2/17) harbored actionable mutations in *PIK3CA* (c.1624G>A, p.E542K, exon 10, and c. 1357G>A, p.E453K, exon 8). An actionable mutation was detected in *ERBB2* (c.929C>A, p.S310Y, exon 8) in a single pattern A tumor (9%, 1/11). One pattern B tumor (6%, 1/17) had an actionable mutation in *KRAS* (c.35G>A, p.G12D, exon 2).

Copy number alterations (CNA)—Copy number gains and amplifications are illustrated in Figure 3. Copy number losses that were identified in relevant gene loci across all patterns in Figure 4. There was a qualitative difference in copy gains from pattern A along the apparent continuum to pattern C (Figure 3). Tumors with patterns A or B showed minimal to no CNA in 5p (*SDHA, RICTOR,* and *TERT),* 5q (*NPM1, FLT4, UIMC1,* and *NSD1),* and 16p (*FUS* and *TSC2)* in comparison to abrupt copy gains in these loci within pattern C HPVAs. A similar trend was observed for chromosomal copy number losses (Figure 4). Namely, pattern A and B HPVAs showed a tendency for loss of the 18q12.3-q21.33 locus (corresponding to *SETBP1*, *SMAD2, SMAD4,* and *BCL2)*, but a salient number of pattern C HPVA demonstrated an extra 'hit' in the form of loss at the 18q arm level, resulting in additional copy loss of *SS18.* Copy number losses in 16q23.2–24.3 (corresponding to *CBFTA, MAF,* and *FANCA*) were evident in patterns A and B, with an additional loss of the 16q arm level in pattern C (corresponding to loss of *CDH1* and *CYLD*).

HPVA overall (all patterns inclusive) showed copy number gains and amplifications in substantial portions of chromosome 3q (corresponding to loci in the *TERC, SOX2,* and *MECOM* genes), 9p (*CDK2NA*), and 21q (*RUNX1*). Additionally, the entire cohort showed copy number losses in substantial portions of 16q (*MAF, CBFA2T3*, and *FANCA*), and 18q (*SETBP1, SMAD2, SMAD4,* and *BCL2*).

Discussion

The relative incidence of cervical adenocarcinoma is increasing worldwide (in part due to the success of cytologic screening for and prevention of cervical squamous cell

carcinomas), rendering efforts towards optimized treatment more important.[16–20] The pattern-based classification of invasive HPVA has emerged as a morphologic analog to historic FIGO staging parameters (tumor size, depth of invasion, horizontal extent), with superior performance in predicting lymph node metastasis and recurrence. Indeed, the schema has been incorporated in the most recent 2020 WHO for Female Genital Tumors,[37] but has yet to be integrated into clinical decision-making algorithms.

As defined and validated in numerous studies, pattern A tumors show well-developed, differentiated glands with a subtle increase in glandular density and complexity beyond the range of non-neoplastic endocervical lobules (some of which conceivably represent adenocarcinoma *in situ*) without destructive invasion or LVI. These have essentially a nil risk of lymph node metastasis, rendering further pelvic node dissection unnecessary. Invasive pattern B is composed of pattern A-type glands that additionally demonstrate incipient and focal destructive invasion in the form of nests or single cells with an accompanying stromal desmoplasia, which can be associated with LVI as well as lymph node metastases (up to 27% and 4%, respectively).[5] Pattern C tumors are characterized as the most aggressive - showing poorly formed, angulated to elongate glands with diffuse and destructive stromal invasion, along with frequent LVI (in up to 62%). Patients in this category frequently present at a higher stage (II) and have an increased risk for lymph node metastasis and recurrence (up to 24% and 22%, respectively) although disparities in overall survival as compared to those of other patterns have yet to be substantiated.[5]

Studies to date have explored molecular distinctions between HPVA and HPV-independent (HPVI) endocervical adenocarcinoma, but there is limited literature evaluating the molecular underpinnings of the various patterns of invasion in HPVA.[11–15] Studies illustrating the genomic landscape of this morphologic continuum were otherwise constrained by either small cohort size and/or limited gene panels. Hodgson et al evaluated 20 HPVA on a 50-gene panel, demonstrating that mutations in *PIK3CA*, *RB1*, and *KRAS* were limited to tumors with ‘destructive’ invasion (ie, patterns B and C). Furthermore, *KRAS* alterations correlated with advanced stage at presentation (II or higher). Due to these apparent molecular similarities, the authors speculate that patterns B and C might in conjunction represent a subset of invasive endocervical adenocarcinoma that are biologically distinct from those displaying an ‘indolent’ pattern (A).[11] Their findings were corroborated in a subsequent study by Spaans et al, who used a 13-gene hot-spot panel in 82 HPVAs to show the topography of alterations in *KRAS*, *PIK3CA*, *PTEN*, and *CDKN2A*. These were, again, proprietary to patterns B and C (save one pattern A with a single *KRAS* mutation).[15] It is also conceivable that destructive patterns of invasion do not arise *de novo* but rather are sequential in nature, developing due to progressive genomic injury from those once classified as indolent. Without longitudinal sampling, documentation of juxtaposed patterns within the same specimen, or subclonal analysis thereof – this premise is difficult to substantiate but provides a working hypothesis for the benefit of larger cohorts.

While the pattern-based classification system as presently structured has been shown to be clinically relevant, it could potentially be further streamlined for clinical use by incorporating other histologic parameters, immunohistochemical profile, and/or underlying genomic aberrations. The concept of sub-stratification based on LVI status was previously

investigated in the context of pattern C tumors, in which the quantity of LVI was associated with lymph node metastases; however, outcome-based analysis showed that lymph node spread, not solely LVI, was correlated with poorer survival.[33]

The findings herein are analogous to prior studies, but with novel and clinically relevant information. In our cohort, *PIK3CA, ERBB2,* and *ATRX* mutations were almost exclusively identified in the provisional 'high-risk' group (pattern B with LVI combined with all pattern C HPVA). While *PIK3CA* and *ERBB2* mutations are well-established in endocervical adenocarcinoma of any type (particularly in comparison to cervical squamous cell carcinoma), the correlation here with destructive invasion reveals a candidate mechanism for the observed histologic and biologic disparities between tumor patterns.[24, 39] *KRAS* and *GNAS* mutations, also common findings in endocervical adenocarcinoma[25] (and in one study possibly associated with more aggressive behavior in both usual (HPVA) and gastric (non-HPVA) types[11]) were found here in similar frequencies throughout the three invasive patterns. There are rare reports of pattern A tumors (and indeed even adenocarcinoma *in situ*) that spread to the ovary via direct extension or metastasis, and in one study 80% and 60% of such cases harbored *KRAS* and/or *GNAS* mutations, respectively.[26, 41] The apparent morphologic 'indolence' of these otherwise aggressive HPVA might be belied by these underlying genomic 'hits' that do not necessarily translate to their histology. Similarly, the frequency of *TP53* mutation – incident in up to 36% of endocervical adenocarcinoma in one study - did not appear to correlate at all with invasive pattern, CNA, or TMB.[38–39] As such it likely does not represent an oncogenic driver mutation in these tumors. Indeed, *TP53* alteration in the context of endocervical adenocarcinoma is more often associated with a gastric mucinous phenotype (ie, HPVI).

Comparing CNA among invasive patterns further supports this overarching concept of tumor progression - ie, acquisition of additional genomic insult reflected in the sequentially evolving histology of stromal infiltration and destruction. Certain chromosomal loci, such as 5p, 5q, and 16p, appeared negligibly altered in 'indolent' (A) or 'minimally destructive' (B) pattern HPVA, but harbored significant gains in a substantial proportion of pattern C HPVA. Interestingly, abnormal segments of 18q and 16q in patterns A and B showed further alterations in pattern C, with completion of loss at the 18q arm level (*SS18*) and 16q arm level (*CDH1* and *CYLD*). Alternatively, it may be speculated that the volumetrically disparate changes observed in aggressive tumors are present from the onset and hence are baseline harbingers of poor behavior. Other loci appeared pan-altered regardless of invasion pattern, including gains of 3q, 9p, and 21q arms along with losses of 16q and 19q.

A particularly unique and heretofore unreported observation is the presence of an APOBEC signature solely in the context of conventionally defined pattern C HPVA. This genetic hallmark is defined by spontaneous deamination of cytidine moieties leading to C>T and C>G aberrations driven in a subset of tumors by viral activation of DNA-editing proteins APOBEC3A/3B. These unscheduled modifications are believed to render a compromised antiviral host defense against oncogenesis. APOBEC-related changes have been described in a variety of human malignancies including those of the breast, lung, pancreas, and bladder (up to 15% of sequenced tumors). As is the interpretation of any genomic 'signature,' APOBEC is neither sensitive nor specific for a unique clinicopathologic circumstance,

but rather is under investigation towards its role as a potential prognostic and therapeutic landmark. Cervical and head/neck carcinomas deriving from HPV infection also frequently harbor this signature,[27–28] and - both here and in other studies - its presence often correlates with a relatively higher TMB.

Accordingly, TMB was significantly elevated in the high-risk group as compared to low-risk tumors, which further supports the concept that pattern consolidation seems to more accurately reflect the underlying biology of these tumors than when grouped in the traditional three patterns. TMB, translated clinically as a surrogate measure of neoantigen load, is directly proportional to the immunogenicity of some tumors and predicts a better response to checkpoint inhibitor therapy.[30–32] As such, it not only reflects the advanced mutagenetic state of high-risk HPVA, but along with the presence of an APOBEC signature can also direct future therapeutic strategies.

Certain limitations to the study are acknowledged. The first involves a relatively modest cohort size, which was extracted from a formerly analyzed sample of endocervical adenocarcinomas that included HPV-associated and HPV-independent forms[12].This selection bias probably contributed to the observation that 40% of recurrent pattern B tumors in our series harbored LVI, which is also disproportionately elevated in comparison to prior studies. Second, as the assignment of pattern was based on the entirely submitted cervix in resection specimens for which the system has been validated, undersampling of destructive invasion is unlikely and this finding is feasibly attributed to other silent genomic and environmental factors. Third, while the expanded gene panel permitted analysis of more genomic targets, the low incidence of otherwise likely significant molecular events could not be evaluated statistically, or – more importantly – associated with outcomes. It is possible that the cohort was underpowered as such. Finally, given the primary objectives, we did not explicitly include adenocarcinoma *in situ* (AIS) in the sequenced cohort. However, further investigation might determine whether there are indeed salient and clinically relevant molecular disparities between the frequently morphologically indistinguishable AIS and invasive pattern A HPVA, thereby warranting their continued separation or move towards consolidation.

To our knowledge thus far, this study represents the most comprehensive genetic investigation of HPVA in the context of invasive pattern, using a 447-gene next generation sequencing panel. As theorized, the genomic changes intrinsic to HPVA were manifest in their degree of destructive invasion as currently defined by the Silva classification. Nonetheless, certain alterations were more closely associated with the recently proposed low-risk (pattern A and pattern B without LVI) and high-risk (patten B with LVI and pattern C) categories. Our study provides genomic evidence in support of this shift towards a binary pattern-based classification, which in tandem with outcome measurements, needs to be further investigated and validated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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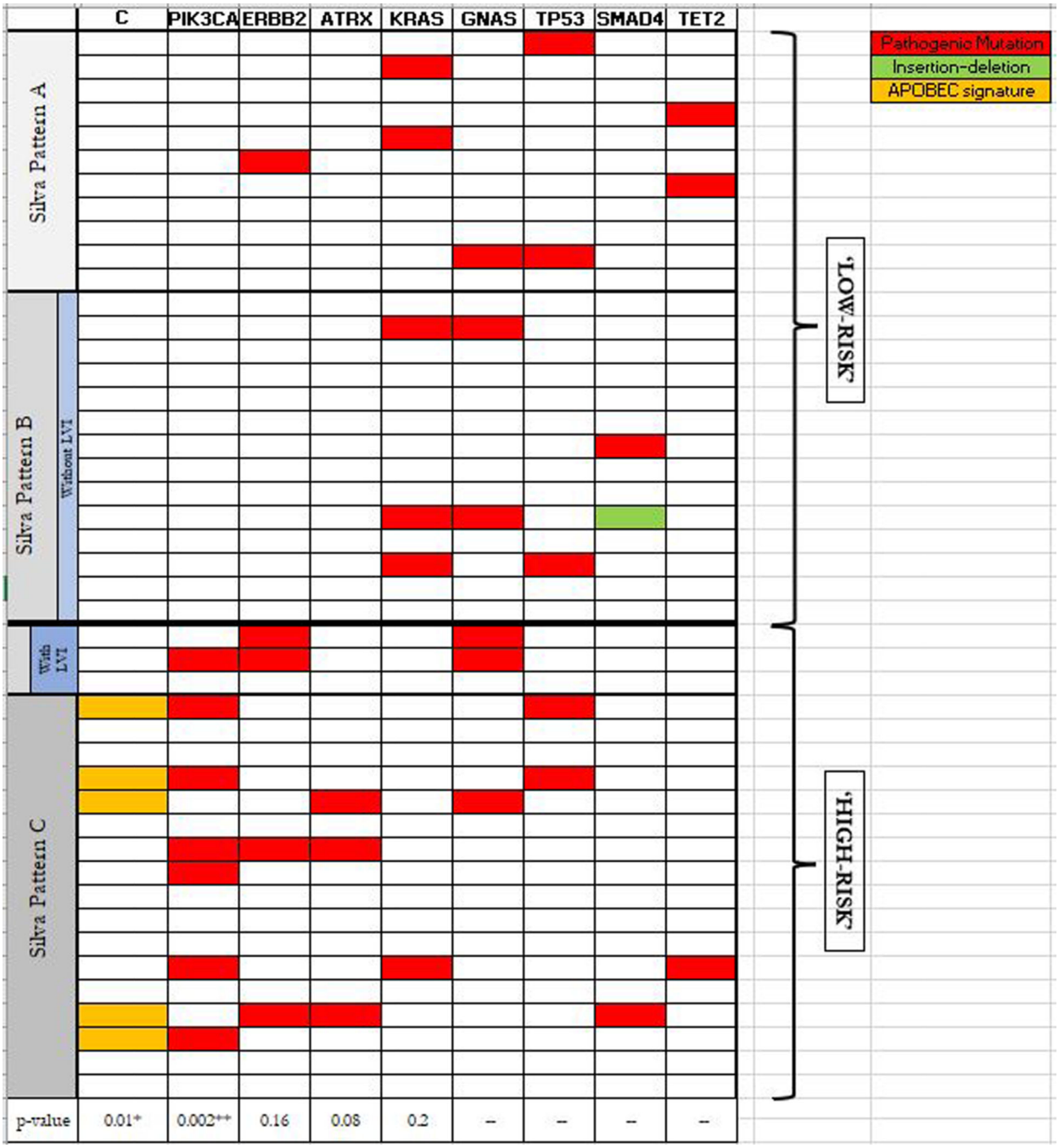

Figure 1:

Pathogenic single nucleotide variations and APOBEC signature, by Silva pattern of invasion. KRAS, GNAS, and TP53 mutations were identified in similar frequency throughout patterns A-C, while PIK3CA, ERBB2, and ATRX mutations were exclusively identified in the 'high-risk' group. ATRX mutations were isolated to pattern C tumors. p-values are shown for comparison between high- and low-risk groups.

	Pattern A	Pattern B	Pattern C	'Low-Risk'	'High-Risk'	Pattern C with APOBEC	Pattern C without APOBEC
Average TMB (mutations/Mb)	5.05	4.56	7.74	4.56	7.53	12.8	5.6
p-value	p=0.14			**p*<0.05**		**p**=0.004**	

Figure 2:

TMB comparison between 1.) the three Silva patterns, 2) low-risk and high-risk tumors, and 3) pattern C tumors with and without APOBEC mutational signaure.

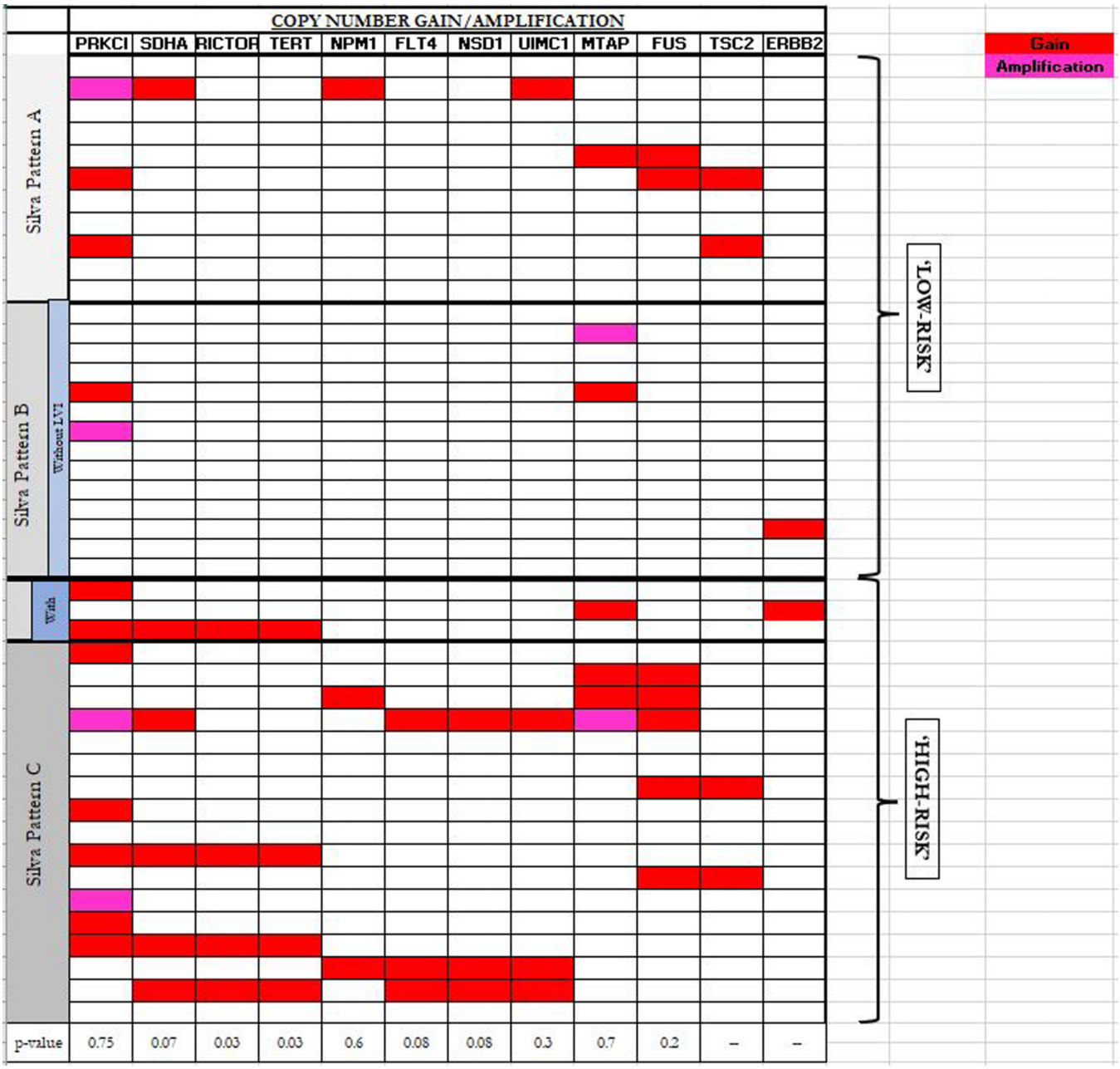

Figure 3:

Copy number gains/amplifications, illustrating the progressive genomic injury from patterns A to C. p-values are shown for comparison between high- and low-risk groups.

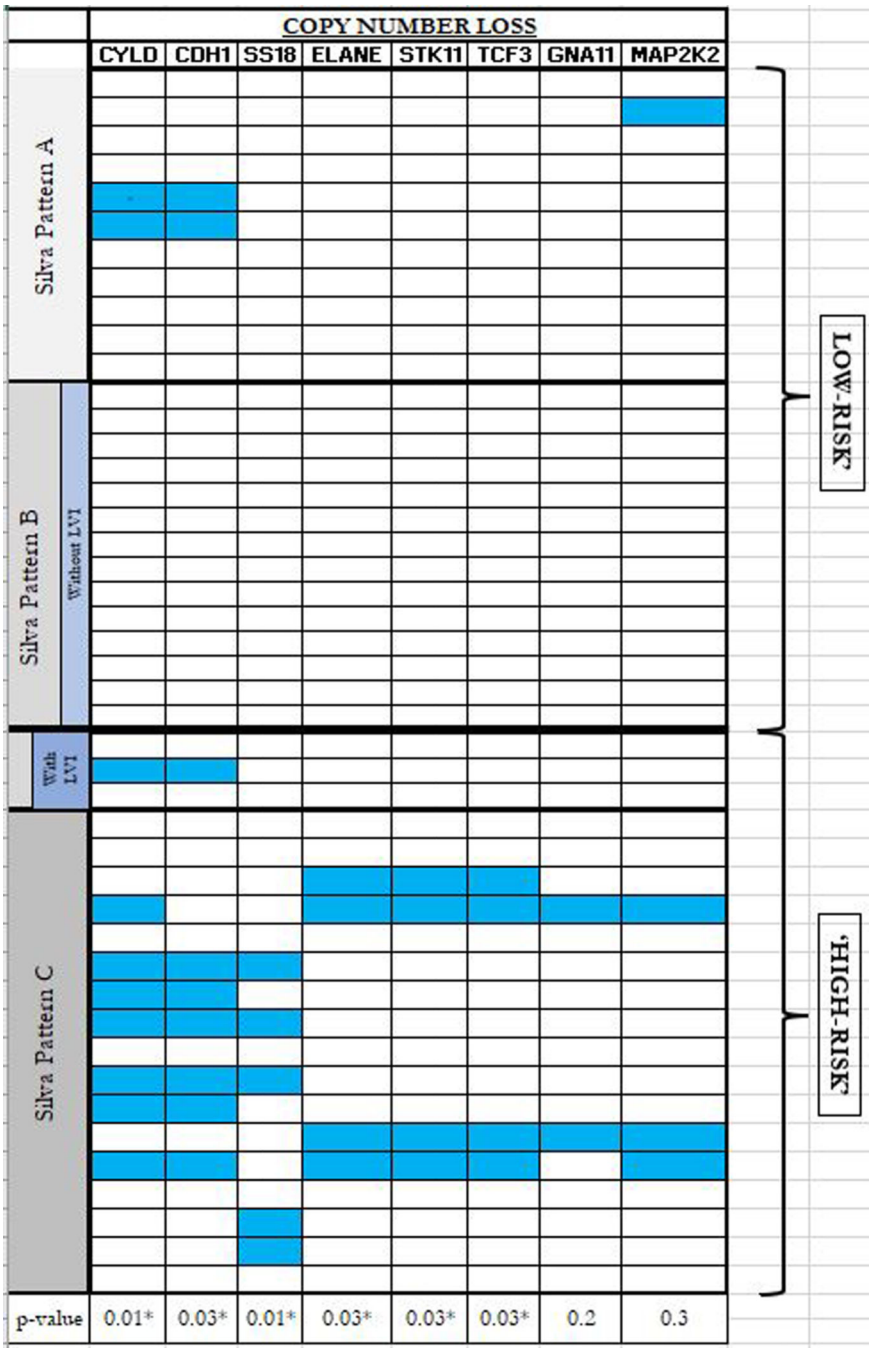

Figure 4:

Copy number losses, observed in greatest volume in pattern C and high-risk tumors. p-values are shown for comparison between high- and low-risk groups.

Table 1:

Clinicopathologic features of ECA cohort by a) Silva pattern of invasion, b) provisional ‘low-risk’ and ‘high-risk’ groups

	n	Age	Stage IA	Stage IB	Stage II	LVI	Lymph Node Metastasis	Recurrence
Pattern A	24% (11/45)	43 ± 17	55% (6/11)	36% (4/11)	9% (1/11)	0% (0/11)	0	0% (0/11)
Pattern B	38% (17/45)	41 ± 10	24% (4/17)	65% (11/17)	12% (2/17)	18% (3/17)	0	29% (5/17)
Pattern C	38% (17/45)	45 ± 16	29% (4/14)	57% (8/14)	14% (2/14)	6% (1/17)	0	12% (2/17)
Low-risk	55% (25/45)	43 ± 13	40% (10/25)	48% (12/25)	12% (3/25)	0% (0/25)	0	12% (3/22)
High-risk	45% (20/45)	44 ± 15	24% (4/17)	65% (11/17)	12% (2/17)	20% (4/20)	0	25% (4/16)