The landscape of somatic mutations in Indonesian cervical cancer is predominated by the PI3K pathway

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Abstract

Objective. To investigate the prevalence of somatic mutations in Indonesian cervical carcinoma patients in the context of histology and human papillomavirus (HPV) type.

Methods. In total 174 somatic hot-spot mutations in 13 genes were analyzed by mass spectrometry in 137 Indonesian cervical carcinomas.

Results. In 66/137 tumors (48%) 95 mutations were identified. *PIK3CA* was most frequently mutated (24%), followed by *FBXW7* (7%), *CTNNB1* (6%), and *PTEN* (6%). In squamous cell carcinomas more often multiple mutations per sample (p=0.040), and more *PIK3CA* (p=0.039) and *CTNNB1* (p=0.038) mutations were detected compared to adenocarcinomas. *PIK3CA* mutations were associated with HPV 16 positivity, *CDKN2A* mutations with HPV 52 positivity, and, interestingly, *PTEN* mutations with HPV negativity. Balinese tumor samples more often carried multiple mutations (p=0.019), and more *CTNNB1, CDKN2A,* and *NRAS* mutations compared to Javanese samples.

Conclusions. Potentially targetable somatic mutations occurred in 48% of Indonesian cervical carcinomas. The landscape of mutations is predominated by mutations concerning the PI3K pathway, and we prompt for more research on developing therapies targeting this pathway, explicitly for the more advanced stage cervical carcinoma patients.

Keywords

cervical carcinoma, somatic mutation, *PIK3CA*, Indonesia, human papillomavirus, cancer genomics

Introduction

Today, around 85% of the global burden of cervical cancer occurs in the least developed countries of the world [1]. In Indonesia, cervical cancer is the second most common cancer in women, with estimated age-standardized incidence and mortality rates (ASR) of 17.3 and 8.2 per 100,000 women per year, respectively. Herewith, the clinical (and economic) burden of this disease in Indonesia is substantial. By contrast, in the Netherlands, cervical cancer is the twelfth most common cancer in women, with an ASR for incidence and mortality of 6.8 and 1.6 per 100,000 women per year, respectively [1].

Cervical cancer is caused by a persistent infection with high risk type human papillomavirus (HPV) [2]. Meta-analyses have shown that HPV type 16 and 18 are responsible for approximately 73% of all cervical cancer cases worldwide, followed by HPV type 58, 33, 45, 31, and 52. However, considerable inter- and intraregional variation of HPV type distribution was described [3, 4]. We have previously investigated the HPV type distribution in the Indonesian population [5] and in Indonesian cervical cancer patients [6], and reported relatively high prevalence rates of HPV type 18 (1.3% population, 38% in cancer) and HPV type 52 (1.8% population, 14% in cancer), and a high percentage of multiple HPV infections (2.3% population, 14% in cancer).

However, with a worldwide overall HPV prevalence of 10% in healthy women, it is known that only a minority of women are prone to develop cervical cancer. The progression from initial infection to a persistent infection into premalignant lesions and eventually invasive cervical cancer is a multifactorial process, influenced by many life-style, environmental, cultural, political, geographical, and socioeconomic factors, such as smoking, parity, age, sexual behavior, and the quality of health care facilities [7]. The differences in incidence and mortality rates for cervical cancer between low-resource and industrialized countries are often ascribed to differences in these factors, and, predominantly, by the (lack of) implementation of cytological screening and/or vaccination programs [8].

In addition, recent studies have shown that various genetic and epigenetic events play an important role in the carcinogenesis of cervical cancer, such as copy number alterations, loss of

heterozygosity, tumor suppressor gene inactivation, or oncogene activation [9-13]. Insight into the molecular mechanisms driving tumorigenesis has become more and more relevant with the emergence of targeted drug therapies. Two well-known examples of successful targeting therapies are trastuzumab for *HER2* overexpressing mamma carcinoma patients, and vamurafenib for *BRAF* mutated melanoma patients [14, 15]. Disappointingly, for cervical cancer, no tumor-specific targeting drugs have proved to be successful yet, though diverse novel agents are enrolled in ongoing clinical trials [16] (https://www.clinicaltrials.gov). Furthermore, the presence or absence of certain somatic mutations in cervical cancer was suggested to be associated with different outcomes to adjuvant chemotherapy treatment and radiation sensitivity [17-19]. Knowledge concerning a tumor's genetic make-up may guide individualized treatment strategies.

Over the past few years, several research groups, including ours, have evaluated the genomic alterations of very small to quite large cohorts of cervical cancer patients [12, 20-26]. And very recently, The Cancer Genome Atlas (TCGA) Research Network published their integrated genomic and molecular characterization of cervical cancer [13]. However, in Indonesia, a high prevalence country for cervical cancer, genetic profiles were never investigated. Whilst preventive vaccines are introduced slowly and with the greatest difficulty [27], still most women present with advanced stage disease. The urge and need for alternative (targeted) adjuvant treatments is greatest in countries like Indonesia, where these treatments seem to be the most faraway though.

In the present study, we analyzed the prevalence of somatic mutations in Indonesian cervical carcinoma patients, and placed this in the context of histology and HPV type. Furthermore, we discussed the similarities and differences in cervical cancer mutation profiles between Indonesian and Dutch cervical cancer patients.

Methods

Patient samples

This study was assessed by the Institutional Review Board. All samples were blinded for patient identification and used according to the Code of Conduct for responsible use of human tissue in the context of health research 2011 (https://www.federa.org/sites/default/files/images/print_version_code_of_conduct_english.pdf).

In total 142 cervical cancer specimens from Indonesia were available. Seventy-four cases derived from the outpatient clinic of the Dr. Cipto Manungkusumo National General Hospital, Jakarta, Java, Indonesia, and consisted of a consecutive cohort of patients diagnosed with invasive cervical cancer (2001-2002) as described previously [6]. An additional 10 Javanese cervical adenocarcinoma samples (2011) were provided from the Santosa Hospital, Bandung, Java, Indonesia. Fifty-eight cases derived from the Sanglah General Hospital, Denpasar, Bali, Indonesia, and consisted of two consecutive cohorts of patients diagnosed with invasive cervical cancer (27 cases from 2009, and 31 cases from 2011).

Of all included patients, formalin-fixed, paraffin-embedded (FFPE) material containing a representative part of the cervical tumor was available at the Leiden University Medical Center. Histological sections were reviewed for morphology by an experienced pathologist (GJF). When no glandular components were seen, sections were stained with Periodic Acid Schiff Plus and Alcian Blue to detect intracytoplasmic mucus. Cases were classified as squamous cell carcinoma (SCC), adenocarcinoma (AC), or adenosquamous carcinoma (ASC) according to the WHO 2014 histological classification of tumors of the uterine cervix [28]. Three samples were excluded from further analysis due to poor fixation or unclear morphology.

All samples included in this study were typed for HPV using the SPF10 primer set and INNO-LiPA HPV genotyping extra line probe assay (Fujirebio Europe, Gent, Belgium) according to the manufacturers protocol.

For DNA isolation, three to five 0.6mm tissue cores were punched out of a marked tumor area of the FFPE tissue block containing >70% tumor. Of some FFPE blocks 10µm tissue sections were taken instead of cores as they contained >70% of tumor cells. DNA was isolated either manually, followed by a DNA purification step (NucleoSpin Tissue kit, Machery-Nagel, Germany), or using the automated Tissue Preparation System (Siemens Healthcare Diagnostics, NY, USA) [29].

After DNA isolation, the FFPE tissue blocks were returned to Indonesia to be stored in the respective local archives.

Mutation Genotyping

The GyneCarta mutation genotyping panel (Agena Bioscience, San Diego) was used to detect 174 known mutations in 13 validated oncogenes and tumor suppressor genes being *BRAF, CDKN2A, CTNNB1, FBXW7, FGFR2, FGFR3, FOXL2, HRAS, KRAS, NRAS, PIK3CA, PPP2R1A,* and *PTEN* [29].

All samples (*N*=142), plus 28 (20%) samples in duplicate and 16/28 in triplicate, four negative controls (H_2O), and two wild type leukocyte DNA samples were genotyped using the iPLEX technology system (Sequenom Inc., San Diego, USA) for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry following the manufacturers' protocol [30]. Two investigators (VS, MT), blinded for tumor identification, analyzed the data independently using Mass Array Typer Analyzer software (TYPER 1.0.22, Sequenom, Hamburg, Germany) and Mutation Surveyor (Softgenetics, State College, Pennsylvania, USA). Two samples failed for all assays (one from Bali, one from Java) and were excluded from further analysis.

Statistics

Statistical analyses were performed with IBM-SPSS Data Editor (version 20.0, Armonk, New York, USA) using the independent Students *t*-Test to compare numerical data and the Chi-squared test or Fisher's exact test to compare categorical and normally distributed data. Pearson's correlation coefficients were used to detect bivariate correlations for HPV positivity or type and mutation status. Binary Logistic regression models were used to perform multivariate analyses for somatic mutation status, or gene specific mutation status, correcting for age, region, histological classification (block 1, method = Enter), and HPV type 16, 18, 52, and 45, and other

gene mutations (block 2, method = Backward Stepwise Conditional). All tests were two-tailed, and p values < 0.05 were considered statistically significant.

Results

Samples

In total 137 samples were analyzed, 82 samples (60%) from Java, 55 samples (40%) from Bali. Tumor characteristics are summarized in table 1. Morphologically, 91 (66%) tumors were classified as SCC, 30 (22%) as AC, and 16 (12%) as ASC. The histological subtypes were unequally distributed amongst the two populations with relatively less SCC, and more AC and ASC in the Javanese cohort (table 1).

In total 120 (88%) samples were HPV positive, with HPV 16 as the most frequently detected HPV type (45%), followed by HPV 18 (29%) and 52 (12%). HPV 39 was the fourth most frequent HPV type, predominantly detected in the Balinese cohort, but occurred in 8/10 cases together with another high risk HPV type. HPV 16 was more frequently detected in SCC compared to AC and ASC (56%, 20%, and 25%, respectively, p=0.001), whereas HPV 18 was more frequently detected in AC and ASC, compared to SCC (53%, 69%, and 14%, respectively, p=0.000). HPV 18 was more frequently detected in Javanese samples, which correlated with the higher frequency of AC and ASCs in this cohort (table 1).

Mutation analyses

In table 2, all detected mutations are listed, and in figures 1 and 2, the mutation spectrum is visualized for single (in grey) and multiple (in black) mutations per gene, for the total cohort, per region, or per histological subtype.

In total, 95 somatic mutations were identified in 66/137 cervical tumors (48%). In 45 tumors (33%) one mutation was detected, in 14 tumors (10%) two mutations were detected, in six tumors (4%)

three mutations were detected, and in one tumor four mutations were detected. Multiple mutations occurred within genes and between genes. *HRAS* mutations occurred significantly more often with a concomitant *CDKN2A* (*N*=2, OR 16.7, 95% CI 1.8-158.8) or *NRAS* mutation (*N*=2, OR 16.7, 95% CI 1.8-158.8).

In the Javanese cohort 44 mutations were detected in 34/82 tumors (42%), in the Balinese cohort 51 mutations were detected in 32/55 tumors (58%) (*p*=0.055). In the Balinese cohort significantly more tumors showed ≥ 2 mutations per sample compared to the Javanese cohort (14/55 (25%) vs. 7/82 (9%), respectively, *p*=0.019). Comparing both cohorts per gene, significant differences were seen between Java and Bali for *CTNNB1* (2% vs. 11%, *p*=0.038), *CDKN2A* (1% vs. 11%, *p*=0.017), and *NRAS* (1% vs. 11%, *p*=0.017).

Comparing by histological subtype, we detected a significantly higher overall mutation frequency in SCC compared to AC (55% vs. 33%, *p*=0.040), and a higher *PIK3CA* mutation frequency in SCC compared to AC (29% vs. 10%, *p*=0.039). No significant differences were seen comparing SCC with ASC, or comparing AC with ASC, taking into account the small number of the ASCs (n=16) in this study. Combining AC and ASC as one subgroup and comparing this with SCC, revealed that *CTNNB1* gene mutations occurred solely in SCC samples (*N*=8 (9%), *p*=0.038).

A correlation analysis was performed to detect associations between age and overall mutation status or gene specific mutation status. No association was found between age an any mutation, nor between age and a *PIK3CA* mutation. However, *CTNNB1* mutations were associated with a significantly higher age at time of diagnosis, with a mean age of 60,3 years in patients with *CTNNB1* mutated tumors, and a mean age of 47,7 years in patients with non-*CTNNB1* mutated tumors (*p*=0.001).

The correlation analysis was repeated for FIGO stage (International Federation of Gynecology and Obstetrics). However, FIGO stages were only known for 66/82 (80%) Javanese tumors (23% stage 1b, 12% 2a, 35% 2b, 30% ≥3a), so this concerns a sub analysis for Javanese samples only. No correlation was found between FIGO stage and a positive mutation status or with multiple mutations. However, *PIK3CA* mutated tumors had significantly higher FIGO stages, with 18/43 FIGO ≥2b tumors mutated (42%) vs. 3/23 FIGO ≤2a tumors mutated (13%) (*p*=0.025). For

all other genes, the mutation rates were too low to perform meaningful statistical analysis, however, within the subgroup of 66 tumors, all mutations in *FBXW7, CTNNB1,* and *KRAS* were seen in FIGO 3b tumors (*N*= 4, 2, and 2, respectively), with no mutations in lower stage tumors. Subsequently, univariate analyses were performed for overall mutation status (having any somatic mutation) or gene specific mutation status, and HPV overall positivity (for any type) or HPV type specific positivity. Results are summarized in table 3. There was a significant correlation between a positive mutation status and a multiple HPV infection (16/21 (76%) vs. 5/21 (24%), *p*=0.006). Furthermore, having any somatic mutation was significantly associated with HPV 16 positivity (36/61 (59%) vs. 25/61 (41%), *p*=0.023), and HPV 52 positivity (12/17 (71%), vs. 5/17 (29%), *p*=0.048). *PTEN* mutations were associated with HPV negativity (4/17 (23%) vs. 4/120 (3), *p*=0.009). *KRAS* mutations were associated with an infection with multiple HPV types (3/21 (14%) vs. 1/99 (1%), *p*=0.017). *PIK3CA* mutations were associated with HPV 16 positivity (23/61 (38%) vs. 10/76 (13%), *p*=0.001), and inversely associated with HPV 18 positivity (5/40 (12%) vs. 28/97 (29%), *p*=0.042). *CDKN2A* mutations correlated with HPV 52 positivity (3/17 (18%) vs. 4/120 (3%), *p*=0.041).

Multivariate analysis

Multivariate logistic regression analyses revealed that having any somatic mutation was associated with HPV 16 (OR 2.5, 95% CI 1.1-5.5), and HPV 52 (OR 4.4, 95% CI 1.3-14.7). Having a *PIK3CA* mutation was associated with HPV 16 (OR 7.9, 95% CI 2.3-27.1) and HPV 45 (OR 12.0, 95% CI 1.6-89.1), not with histological subtype or age. Having a *CTNNB1* mutation was associated with age (OR 1.1, 95% CI 1.0-1.2), not with histopathology, nor with Balinese origin. Having a *CDKN2A* mutation was associated with HPV 52 (OR 30.8, 95% CI 1.9-489.3), with a concomitant *HRAS* mutation (OR 38.7, 95% CI 1.3-1157.8), and AC subtype (OR 27, 95% CI 1.1-674.4), but not with Balinese origin (OR 13.3, 95% CI 0.68-258.8). However, having a *NRAS* mutation was associated with Balinese origin (OR 10.7, 95% CI 1.0-113.3), and also with a concomitant *HRAS* mutation (OR 15.3, 95% CI 1.5-155.5). The other way around, *HRAS*

mutations were associated with a concomitant *CDKN2A* or *NRAS* mutation (OR 18.5 and 12.5, 95% CI 1.4-252.2 and 1.2-135.4, respectively).

Discussion

In the present study, we have shown that potentially actionable somatic mutations occurred in 48% of Indonesian cervical carcinomas. The landscape of mutations showed similarities as well as differences between two Indonesian cancer cohorts from Java and Bali, and several correlations were shown between somatic mutations and HPV (type) positivity.

With the emergence of tumor targeting drugs such as tyrosine kinase inhibitors, targeting the tumor based on its genomic profile rather than its histological background, it is important to study the prevalence of targetable oncogenic driver mutations throughout diverse ethnical cancer populations from diverse geographic areas. The prevalence of somatic mutations in cervical cancer was investigated previously in other cervical cancer cohorts worldwide from the US (*N*=80) [21], Norway/Mexico (*N*=100/15) [12], China (*N*=285) [23], the Netherlands (*N*=301) [20], France (*N*=29) [25], Hong Kong (*N*=15) [22], Guatemala/Venezuela/Mexico (*N*=280/40/325) [24], to India (*N*=10) [26], using varying techniques, from whole genome and/or exome sequencing [12, 13, 26], direct sequencing [23], to oncopanel analysis [20, 21, 25] or a combination of techniques [22, 24].

This is the first study to describe the prevalence of driver mutations in an Indonesian cervical cancer cohort. Indonesia is the world's largest, and most widely scattered archipelago, populated by more than 260 million people of more than 300 distinct native ethnic groups, and where cervical cancer is still the second most common cancer in women [1]. We analyzed a Javanese cohort, representing the largest ethnical Muslim population derived from the island Java, and compared this with a Balinese cohort, representing a relatively isolated Hindu population from the island Bali. We described the similarities and differences of the mutation spectrum for both cohorts (figure 1), and in multivariate analysis, a significantly higher mutation frequency of *NRAS* was seen in Balinese- (11%) compared to Javanese patients (1%). This is the first cervical cancer

cohort in which a *NRAS* mutation rate of 11% was described, and this may be of interest for future targeted therapies. *NRAS* plays a role in PI3K as well as MAPK signaling and is mutated in 15-20% of melanomas. Studies concerning NRAS mutated melanomas suggested that combined targeting of both pathways may improve treatment [31].

Recently, we have reported on the mutation spectrum of a Dutch cervical cancer cohort [20], using the same mutation panel as in the present study [29], and therefore, comparisons between Indonesia, a high incidence country, and the Netherlands, a low incidence country, could be performed. A significantly higher overall mutation frequency, as well as a higher rate of multiple mutations per sample, and significantly more *FBXW7*, *CDKN2A*, *NRAS*, and *HRAS* mutations were seen in the Indonesian cohort compared to the Dutch cohort (supplementary table 1). It remains uncertain whether these differences are attributable to race/ethnicity/geography, or that they are based on differences in tumor characteristics or stage.

One limitation of the present study is the lack of some relevant clinicopathological characteristics of the Indonesian samples such as FIGO stage, tumor diameter, lymph node metastasis, and survival. However, FIGO stage data were known for 66 Javanese patients, and showed significantly more advanced stage disease compared to the Dutch cohort (Indonesian cohort 20/66 (30%) ≥ FIGO stage 3a, whilst Dutch cohort consisted of only stage 1b-2b tumors, $p<0.001$). We presume, this could also be the case for the Balinese patients, as it is known that in Indonesia, patients often present with advanced stage disease. It is hypothesized that cancer, including cervical cancer, results from sequential mutations in specific oncogenes and/or tumor suppressor genes, and that the mutation frequency increases with advanced cancer stage [32]. However, in the present study, we found no association between increasing FIGO stage and overall mutation frequency or multiple mutations, which is in line with other reports [12, 13, 21, 23, 24]. Gene specifically, however, we do see that the occurrence of *PIK3CA* mutations is associated with higher FIGO stage tumors, which is in line with the Dutch cohort [20] and a recently published study by Verlaat et al., showing that*PIK3CA* mutations are considered a late event in cervical carcinogenesis, and a rare event in its precursor lesions [33].

PIK3CA was the most frequently mutated gene (24%) in the present Indonesian cervical cancer cohort, which is in line with previous reported frequencies in cervical cancer from the Netherlands (20%), France (27%), Latin America (28-33%), the U.S. (31%), and the TCGA data (26%) [13, 20, 21, 24, 25]. However, lower frequencies were also described in Norway (15%) and China (12%) [12, 23]. And in a recent study from India, whole exome sequencing was performed on 10 FIGO stage 3b SCCs, with no *PIK3CA* mutations detected at all [26]. *PIK3CA* mutations lead to an altered production of the catalytic subunit p110α of the enzyme phosphatidylinositol 3-kinase (PI3K), allowing the PI3K pathway to signal without regulation, leading to uncontrolled cell growth, proliferation and survival. The tumor suppressor *PTEN* was third most frequently mutated in Indonesian cervical cancer (6%), comparable with the mutation frequency of Dutch (4%) and Norwegian (6%) cervical cancer patients, and the TCGA data (8%) [12, 13, 20]. The function of PTEN is to dephosphorylate PI3K, and mutations lead to uncontrolled cell growth. *PIK3CA* and *PTEN* are the most frequently mutated genes in human cancers, and therapeutics targeting the PI3K pathway are being developed rapidly, and are today in diverse phases of (pre)clinical trials [34]. Though, for cervical cancer, therapies targeting the PI3K pathway are still scarce [35].

In the Indonesian cohort, 97% of the *PIK3CA* mutated tumors were mutated in the helical domain, dominated by p.E545K, and followed by p.E542K; only 2 mutations in the kinase domain were detected (p.H1047L and p.H1047Y), which is in line with other studies [13, 24]. This is a distinctive feature of cervical carcinoma compared to other cancers with high frequencies of *PIK3CA* mutations, such as endometrial, ovarian, breast, and colorectal carcinoma, in which mutations in the kinase domain occur at least as frequent in the helical domain [24]. Unfortunately, it is the kinase domain H1047R mutation that is explicitly associated with an increased response rate to PI3K/AKT/mTOR inhibitors [36]. In a study of Wang et al., 15/60 locally advanced cervical SCCs had E542K or E545K mutations (there were no kinase domain mutations), and these patients showed a significantly worse response to cisplatinum based chemoradiation [17]. Further research is necessary to develop therapies that can intervene cancers with specific *PIK3CA* helical domain mutations.

The p53-dependent tumor suppressor gene *FBXW7* also plays a role in the PI3K/mTOR pathway, and was the second most frequently mutated gene (6%) in Indonesian cervical cancer, which was significantly more frequent compared to our Dutch cohort (1%), but less compared to the study of Ojesina et al. (15%), and the TGCA data (11%) [12, 13, 20]. *FBXW7* mutations are hypothesized to be a late event in cervical cancer, which might explain the higher frequency in the Indonesian cohort [32]. *FBXW7* mutated tumor cell lines have shown to be sensitive to rapamycin treatment, and we urge for further research concerning *FBXW7* mutated cervical carcinomas [37].

Furthermore, in this study we compared the mutation frequencies between histological subgroups, as we determined some differences between SCC, AC, and ASC in our previous study concerning Dutch cervical carcinomas [20]. In accordance with our study concerning Dutch carcinomas, also in Indonesia *PIK3CA* mutations and *CTNNB1* mutations were associated with the SCC subtype. However, in the TCGA data, *CTNNB1* mutations were only detected in three samples (1,7%) of which two were SCC subtype and one was a AC [13]. Remarkable, *KRAS* mutations were not associated with AC in the Indonesian cohort, which is in contrast with many other studies [12, 13, 20, 21].

We also investigated the presence of HPV and its correlations with somatic mutations. In Indonesia, a different HPV type distribution amongst the population as well as in cervical cancer patients was described, especially with a significantly higher prevalence rate of HPV 52 [5, 6]. In a recent large, retrospective cohort study from Murdiyarso et al., 11.224 cytology swabs from Jakarta area were typed for HPV, and HPV 52 was the most prevalent HPV type in normal cytology (1%), and the second most common type in SCC (26%) [38]. It is unclear why no AC were included in that study. In the current cohort, again we showed a remarkable high prevalence of HPV 52 in the Javanese (12%) as well as in the Balinese cohort (13%). This is an important finding in the light of preventive strategies, because HPV 52 is not included in the available FDA approved HPV vaccines yet.

Significant associations were identified between the presence of any somatic mutation and HPV 16 positivity, based on the positive correlation between *PIK3CA* mutations and HPV 16 positivity. In the Dutch cervical cancer cohort this association was not found. Also Wright et al. investigated

associations between HPV type and *PIK3CA* or *KRAS* mutations in cervical carcinomas, but did not detect any [21]. Contrary, *PTEN* mutations were associated with HPV negativity, a feature that was also seen in the Dutch cohort, and described previously by Minaguchi et al.[39]. However, the coverage of possible *PTEN* mutations by the mutation panel used was only 40% [29]. Therefore, additional techniques such as immunohistochemistry should be performed to identify the true mutation rate of *PTEN* in cervical cancer, to clarify its association with HPV. We also detected an association between any somatic mutation and HPV 52 positivity, based on the positive correlation between *CDKN2A* mutations and HPV 52 positivity. *CDKN2A* was mutated in 11% of the Balinese cervical carcinoma patients, which is the highest frequency described in cervical cancer compared to other studies [20, 24]. Its correlation with HPV 52 is remarkable, and has not been described previously. Given the high prevalence rates of HPV 52 in Indonesia this feature certainly warrants for further investigation.

To conclude, we have presented the landscape of potentially actionable somatic mutations in an Indonesian cervical cancer cohort, and placed the results in the context of histology and HPV type. Most noticeable is the predominance of mutations concerning the PI3K pathway, in concordance with results from other countries. Although we realize that implementation of expensive targeting therapies in countries like Indonesia remains highly uncertain, we do prompt for more research to develop therapies that target this PI3K pathway, explicitly for more advanced stage cervical carcinoma patients.

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Conflict of Interest Statement

The authors have nothing to disclose.

References

[1] Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, et al. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. p. Available from: http://globocan.iarc.fr, accessed on 29/12/2016.

[2] zur Hausen H. Papillomaviruses in the causation of human cancers - a brief historical account. Virology. 2009;384:260-5.

[3] Guan P, Howell-Jones R, Li N, Bruni L, de Sanjose S, Franceschi S, et al. Human papillomavirus types in 115,789 HPV-positive women: a meta-analysis from cervical infection to cancer. Int J Cancer. 2012;131:2349-59.

[4] Li N, Franceschi S, Howell-Jones R, Snijders PJ, Clifford GM. Human papillomavirus type distribution in 30,848 invasive cervical cancers worldwide: Variation by geographical region, histological type and year of publication. Int J Cancer. 2011;128:927-35.

[5] Vet JN, de Boer MA, van den Akker BE, Siregar B, Lisnawati, Budiningsih S, et al. Prevalence of human papillomavirus in Indonesia: a population-based study in three regions. Br J Cancer. 2008;99:214-8.

[6] Schellekens MC, Dijkman A, Aziz MF, Siregar B, Cornain S, Kolkman-Uljee S, et al. Prevalence of single and multiple HPV types in cervical carcinomas in Jakarta, Indonesia. Gynecol Oncol. 2004;93:49-53.

[7] Castellsague X, Munoz N. Chapter 3: Cofactors in human papillomavirus carcinogenesis--role of parity, oral contraceptives, and tobacco smoking. J Natl Cancer Inst Monogr. 2003:20-8.

[8] Domingo EJ, Noviani R, Noor MR, Ngelangel CA, Limpaphayom KK, Thuan TV, et al. Epidemiology and prevention of cervical cancer in Indonesia, Malaysia, the Philippines, Thailand and Vietnam. Vaccine. 2008;26 Suppl 12:M71-9.

[9] Wang SS, Hildesheim A. Chapter 5: Viral and host factors in human papillomavirus persistence and progression. J Natl Cancer Inst Monogr. 2003:35-40.

[10] Narayan G, Murty VV. Integrative genomic approaches in cervical cancer: implications for molecular pathogenesis. Future Oncol. 2010;6:1643-52.

[11] Saavedra KP, Brebi PM, Roa JC. Epigenetic alterations in preneoplastic and neoplastic lesions of the cervix. Clin Epigenetics. 2012;4:13.

[12] Ojesina AI, Lichtenstein L, Freeman SS, Pedamallu CS, Imaz-Rosshandler I, Pugh TJ, et al. Landscape of genomic alterations in cervical carcinomas. Nature. 2014;506:371-5.

[13] Cancer Genome Atlas Research N. Integrated genomic and molecular characterization of cervical cancer. Nature. 2017.

[14] Mates M, Fletcher GG, Freedman OC, Eisen A, Gandhi S, Trudeau ME, et al. Systemic targeted therapy for her2-positive early female breast cancer: a systematic review of the evidence for the 2014 Cancer Care Ontario systemic therapy guideline. Curr Oncol. 2015;22:S114-22.

[15] McArthur GA, Chapman PB, Robert C, Larkin J, Haanen JB, Dummer R, et al. Safety and efficacy of vemurafenib in BRAF(V600E) and BRAF(V600K) mutation-positive melanoma (BRIM-3): extended follow-up of a phase 3, randomised, open-label study. Lancet Oncol. 2014;15:323-32.

[16] Boussios S, Seraj E, Zarkavelis G, Petrakis D, Kollas A, Kafantari A, et al. Management of patients with recurrent/advanced cervical cancer beyond first line platinum regimens: Where do we stand? A literature review. Crit Rev Oncol Hematol. 2016;108:164-74.

[17] Wang J, Chai YL, Wang T, Liu JH, Dai PG, Liu Z. Genetic alterations of PIK3CA and tumor response in patients with locally advanced cervical squamous cell carcinoma treated with cisplatin-based concurrent chemoradiotherapy. Exp Mol Pathol. 2015;98:407-10.

[18] Hou MM, Liu X, Wheler J, Naing A, Hong D, Bodurka D, et al. Outcomes of patients with metastatic cervical cancer in a phase I clinical trials program. Anticancer Res. 2014;34:2349-55.

[19] Xia S, Zhao Y, Yu S, Zhang M. Activated PI3K/Akt/COX-2 pathway induces resistance to radiation in human cervical cancer HeLa cells. Cancer Biother Radiopharm. 2010;25:317-23.

[20] Spaans VM, Trietsch MD, Peters AA, Osse M, Ter Haar N, Fleuren GJ, et al. Precise Classification of Cervical Carcinomas Combined with Somatic Mutation Profiling Contributes to Predicting Disease Outcome. PLoS One. 2015;10:e0133670.

[21] Wright AA, Howitt BE, Myers AP, Dahlberg SE, Palescandolo E, Van Hummelen P, et al. Oncogenic mutations in cervical cancer: genomic differences between adenocarcinomas and squamous cell carcinomas of the cervix. Cancer. 2013;119:3776-83.

[22] Chung TK, Van Hummelen P, Chan PK, Cheung TH, Yim SF, Yu MY, et al. Genomic aberrations in cervical adenocarcinomas in Hong Kong Chinese women. Int J Cancer. 2015;137:776-83.

[23] Xiang L, Li J, Jiang W, Shen X, Yang W, Wu X, et al. Comprehensive analysis of targetable oncogenic mutations in chinese cervical cancers. Oncotarget. 2015;6:4968-75.

[24] Lou H, Villagran G, Boland JF, Im KM, Polo S, Zhou W, et al. Genome Analysis of Latin American Cervical Cancer: Frequent Activation of the PIK3CA Pathway. Clin Cancer Res. 2015;21:5360-70.

[25] Muller E, Brault B, Holmes A, Legros A, Jeannot E, Campitelli M, et al. Genetic profiles of cervical tumors by high-throughput sequencing for personalized medical care. Cancer Med. 2015;4:1484-93.

[26] Das P, Bansal A, Rao SN, Deodhar K, Mahantshetty U, Shrivastava SK, et al. Somatic Variations in Cervical Cancers in Indian Patients. PLoS One. 2016;11:e0165878.

[27] Prayudi PK, Permatasari AA, Winata IG, Suwiyoga K. Impact of human papilloma virus vaccination on adolescent knowledge, perception of sexual risk and need for safer sexual behaviors in Bali, Indonesia. J Obstet Gynaecol Res. 2016;42:1829-38.

[28] Wells M, Ostor AG, Crum CP, Franceschi S, Tommasino M, Nesland JM, et al. Tumours of the Uterine Cervix. In: Kurman RJ, Carcangiu ML, Herrington CS, Young RH, editors. WHO Classification of Tumours of Female Reproductive Organs. 4th ed. Lyon, France: IARC; 2014. p. 169-207.

[29] Spaans VM, Trietsch MD, Crobach S, Stelloo E, Kremer D, Osse EM, et al. Designing a high-throughput somatic mutation profiling panel specifically for gynaecological cancers. PLoS One. 2014;9:e93451.

[30] Fumagalli D, Gavin PG, Taniyama Y, Kim SI, Choi HJ, Paik S, et al. A rapid, sensitive, reproducible and cost-effective method for mutation profiling of colon cancer and metastatic lymph nodes. BMC Cancer. 2010;10:101.

[31] Johnson DB, Puzanov I. Treatment of NRAS-mutant melanoma. Curr Treat Options Oncol. 2015;16:15.

[32] Vogelstein B, Kinzler KW. The Path to Cancer --Three Strikes and You're Out. N Engl J Med. 2015;373:1895-8.

[33] Verlaat W, Snijders PJ, van Moorsel MI, Bleeker M, Rozendaal L, Sie D, et al. Somatic mutation in PIK3CA is a late event in cervical carcinogenesis. J Pathol Clin Res. 2015;1:207-11.

[34] Courtney KD, Corcoran RB, Engelman JA. The PI3K pathway as drug target in human cancer. J Clin Oncol. 2010;28:1075-83.

[35] Bregar AJ, Growdon WB. Emerging strategies for targeting PI3K in gynecologic cancer. Gynecol Oncol. 2016;140:333-44.

[36] Janku F, Wheler JJ, Naing A, Falchook GS, Hong DS, Stepanek VM, et al. PIK3CA mutation H1047R is associated with response to PI3K/AKT/mTOR signaling pathway inhibitors in early-phase clinical trials. Cancer Res. 2013;73:276-84.

[37] Mao JH, Kim IJ, Wu D, Climent J, Kang HC, DelRosario R, et al. FBXW7 targets mTOR for degradation and cooperates with PTEN in tumor suppression. Science. 2008;321:1499-502.

[38] Murdiyarso LS, Kartawinata M, Jenie I, Widjajahakim G, Hidajat H, Sembiring R, et al. Single and multiple high-risk and low-risk Human Papillomavirus association with cervical lesions of 11,224 women in Jakarta. Cancer Causes Control. 2016;27:1371-9.

[39] Minaguchi T, Yoshikawa H, Nakagawa S, Yasugi T, Yano T, Iwase H, et al. Association of PTEN mutation with HPV-negative adenocarcinoma of the uterine cervix. Cancer Lett. 2004;210:57-62.

Table legends

Table 1. *Baseline characteristics*

Baseline characteristics of all 137 included cervical carcinoma patients from Indonesia, and for the Javanese and Balinese cohorts separately. *P* values in bold were considered to indicate statistical significance. Abbreviations: *N,* number; IQR, interquartile range; SCC, squamous cell carcinoma; AC, adenocarcinoma; ASC, adenosquamous carcinoma; HPV, human papillomavirus. * Other, infrequent, HPV types detected were the high risk types HPV 31 (*N*=2), 33 (*N*=2), 35 (*N*=1), 51 (*N*=1), 56 (*N*=1), 58 (*N*=1), 59 (*N*=2), 66 (*N*=1), and "X" (*N*=1), and the low risk types HPV 11 (*N*=1), and HPV 54 (*N*=1). The low risk HPV types occurred concomitantly with HPV 16 and with HPV 33 and 52, respectively.

Table 2. *Mutation frequencies*

Mutation frequencies as detected in a cohort of 137 Indonesian cervical cancer samples. In total 174 hot spot mutations in 13 genes were analyzed. Mutations are shown per gene and in order of frequency, mutations of genes that were not detected in any of the samples are not shown. *BRAF, FGFR2, and FOXL2* genes are not listed because no mutations were detected. *N,* number of samples with the mutation; %, percentage of mutated samples of 137 cervical cancer samples. [a] Three samples contained two *PIK3CA* mutations (2x E542K with E545K, and 1x E545K with H1047Y); [b] One sample contained two *CTNNB1* mutations (T41A with G34E); [c] One sample contained two *PTEN* mutations (R130fs*4 with Q214*).

Table 3. *Correlations between human papillomavirus infection and mutations*

Correlations between human papillomavirus (HPV) infection (any, multiple, or type specific), and somatic mutations (any, multiple, gene specific) are shown in number of HPV positive samples being mutated (percentage between brackets). Numbers and percentages in bold indicate statistical significant correlations. Two-sided *p* values were calculated by Chi-squared test or Fishers' exact test and only significant *p* values are annotated in the present table.

Figure legends

Figure 1. *Mutation spectrum per region*

Spectrum of somatic mutations detected in 137 Indonesian cervical cancer specimen (top panel) and with separate spectra for the Javanese and Balinese cohorts (middle and bottom panel, respectively) in *N,* number of mutated samples, and %, percentage of mutated samples within the cohort. The spectra are visualized from left to right in percentages, with black bars indicating samples with ≥2 mutations, and grey bars indicating samples with 1 mutation.

Figure 2. *Mutation spectrum per histological subtype*

Spectrum of somatic mutations detected in 137 Indonesian cervical cancer specimen (see also figure 1) separately visualized for squamous cell carcinomas (SCC, top panel), adenocarcinomas (AC, middle panel), and adenosquamous carcinomas (ASC, bottom panel) in *N*, number of mutated samples, and %, percentage of mutated samples within the cohort. The spectra are visualized from left to right in percentages, with black bars indicating samples with ≥2 mutations, and grey bars indicating samples with 1 mutation.

Supplementary Information

Supplementary Table S1. *Comparison of mutation frequencies between Indonesia and the Netherlands*

A cohort of 301 consecutive Dutch cervical carcinomas (166 squamous cell carcinomas, 55 adenocarcinomas, and 80 adenosquamous carcinomas) was analyzed for somatic mutations previously using the Gynecarta mutation panel, as described by Spaans et al. [20]. Mutation data were compared with the current Indonesian cervical cancer cohort of 137 carcinomas.

Highlights

- In 48% of 137 Indonesian cervical carcinomas ≥ 1 somatic mutation is present
- Most frequently mutated are *PIK3CA* (24%), *FBXW7* (7%), *CTNNB1* (6%), and *PTEN* (6%)
- Squamous cell carcinomas show more *PIK3CA* and *CTNNB1* mutations than adenocarcinomas
- *PIK3CA* mutations correlate with HPV16, *CDKN2A* – with HPV52, *PTEN* – with HPV absence
- Prioritize research of PI3K-pathway targeting therapies in advanced cervical cancer

564 **Table 1**. Baseline characteristics

		Total *N*=137	Java *N*=82	Bali *N*=55	*p* value Java vs. Bali
Age in years, median (IQR)		47 (41-53)	46 (41-52)	49 (41-58)	0.057
Morphology, *N* (%)	SCC	91 (66)	45 (55)	45 (84)	**0.002**
	AC	30 (22)	25 (31)	5 (9)	
	ASC	16 (12)	12 (15)	4 (7)	
HPV positive, *N* (%)		120 (88)	77 (94)	43 (78)	**0.006**
>1 HPV type detected, *N* (%)		21 (15)	10 (12)	11 (20)	**0.006**
HPV type distribution, *N* (%)	HPV 16	61 (45)	33 (40)	28 (51)	0.218
	HPV 18	40 (29)	32 (39)	8 (15)	**0.002**
	HPV 52	17 (12)	10 (12)	7 (13)	0.926
	HPV 39	10 (7)	1 (1)	9 (16)	**0.001**
	HPV 45	6 (3)	5 (6)	1 (2)	0.401
	Other*	14 (10)	8 (7)	6 (11)	

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Table 2. Mutation frequencies

Gene/mutation	*N*	%
PIK3CA [a]	33	24.1
p.E545K	27	
p.E542K	6	
p.E545D	1	
p.H1047L	1	
p.H1047Y	1	
FBXW7	9	6.6
p.R465H	4	
p.R465C	2	
p.R479Q	2	
p.R479L	1	
CTNNB1 [b]	8	5.8
p.G34E	5	
p.G34R	2	
p.S33Y	1	
p.T41A	1	
PTEN [c]	8	5.8
p.R130fs*4	6	
p.R173H	1	
p.Q214*	1	
p.L318fs*2	1	
CDKN2A	7	5.1
p.W110*	3	
p.R58*	2	
p.P114L	2	
NRAS	7	5.1
p.G12S	2	
p.G13D	2	
p.G12D	1	
p.G12V	1	
p.Q61K	1	
PPP2R1A	7	5.1
p.R258H	7	
KRAS	5	3.6
p.G12A	2	
p.G12D	2	
p.G12V	1	
HRAS	5	3.6
p.G13S	3	
p.G12S	1	
p.G13D	1	
FGFR3	1	0.7
p.A391E	1	

570 **Table 3.** Correlations between human papillomavirus infection and mutations

HPV positive for: Gene mutation:	any type (*N*=120)	≥2 types (*N*=21)	type 16 (*N*=61)	type 18 (*N*=40)	type 52 (*N*=17)	type 39 (*N*=10)	type 45 (*N*=6)
Any mutation (*N*=66)	59 (49)	**16 (76)[a]**	**36 (59)[b]**	16 (40)	**12 (71)[c]**	6 (60)	3 (50)
≥2 mutations (*N*=21)	18 (31)	5 (31)	12 (33)	5 (31)	3 (25)	2 (33)	0 (0)
PIK3CA (*N*=33)	32 (27)	7 (33)	**23 (38)[f]**	**5 (13)[g]**	5 (29)	0 (0)	3 (50)
FBXW7 (N=9)	7 (6)	2 (10)	4 (7)	2 (5)	1 (6)	1 (10)	0 (0)
CTNNB1 (N=8)	7 (6)	3 (14)	4 (7)	2 (5)	2 (12)	1 (10)	0 (0)
PTEN (N=8)	**4 (3)[d]**	1 (5)	3 (5)	1 (3)	1 (6)	1 (10)	0 (0)
CDKN2A (N=7)	7 (6)	2 (10)	2 (3)	3 (8)	**3 (18)[h]**	2 (20)	0 (0)
NRAS (N=7)	7 (6)	1 (5)	5 (8)	2 (5)	1 (6)	1 (10)	0 (0)
PPP2R1A (N=7)	6 (5)	1 (5)	4 (7)	2 (5)	0 (0)	0 (0)	0 (0)
KRAS (N=5)	4 (3)	**3 (14)[e]**	2 (3)	2 (5)	2 (12)	0 (0)	0 (0)
HRAS (N=5)	5 (4)	1 (5)	2 (3)	2 (5)	1 (6)	1 (10)	0 (0)
FGFR3 (N=1)	1 (1)	1 (5)	1 (2)	1 (3)	0 (0)	1 (10)	0 (0)
p value		**[a] 0.006**	**[b] 0.023**		**[c] 0.048**		
	[d] 0.009	**[e] 0.017**	**[f] 0.001**	**[g] 0.042**	**[h] 0.041**		

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573 **Table S1.** Comparison of mutation frequencies between Indonesia and the Netherlands

Gene mutation:	Indonesia *N*=137	The Netherlands* *N*=301	*p* value
Any mutation	66 (48%)	103 (34%)	0.005
≥2 mutations	21 (15%)	13 (4%)	0.002
PIK3CA	33 (24%)	61 (20%)	ns
FBXW7	9 (7%)	3 (1%)	0.002
CTNNB1	8 (6%)	8 (3%)	ns
PTEN	8 (6%)	12 (4%)	ns
CDKN2A	7 (5%)	4 (1%)	0.019
NRAS	7 (5%)	1 (<1%)	0.001
PPP2R1A	7 (5%)	9 (3%)	ns
KRAS	5 (4%)	20 (7%)	ns
HRAS	5 (4%)	1 (<1%)	0.012
FGFR3	1 (1%)	2 (1%)	ns

574 * Cohort described previously by Spaans et al. (2015) in PLoS ONE 10(7):e013670.

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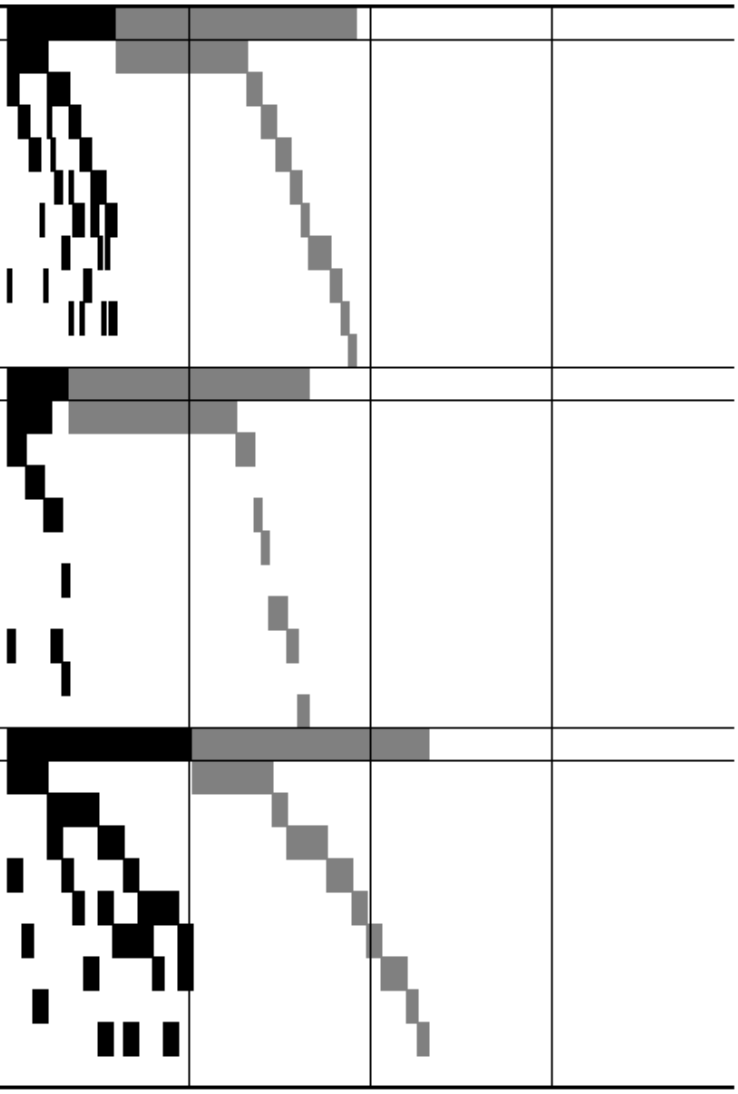
Region	Gene	*N*	%	Mutation Spectrum
				25% 50% 75% 100%
Total *N* = 137	Any Mutation	66	48.2	
	PIK3CA	33	24.1	
	FBXW7	9	6.6	
	CTNNB1	8	5.8	
	PTEN	8	5.8	
	CDKN2A	7	5.1	
	NRAS	7	5.1	
	PPP2R1A	7	5.1	
	KRAS	5	3.6	
	HRAS	5	3.6	
	FGFR3	1	0.7	
Java *N* = 82	Any Mutation	34	41.5	
	PIK3CA	24	29.3	
	FBXW7	4	4.9	
	CTNNB1	2	2.4	
	PTEN	3	3.7	
	CDKN2A	1	1.2	
	NRAS	1	1.2	
	PPP2R1A	2	2.4	
	KRAS	3	3.7	
	HRAS	1	1.2	
	FGFR3	1	1.2	
Bali *N* = 55	Any Mutation	32	58.2	
	PIK3CA	9	16.4	
	FBXW7	5	9.1	
	CTNNB1	6	10.9	
	PTEN	5	9.1	
	CDKN2A	6	10.9	
	NRAS	6	10.9	
	PPP2R1A	5	9.1	
	KRAS	2	3.6	
	HRAS	4	7.3	
	FGFR3	0	0.0	

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Histology	Gene	*N*	%	Mutation Spectrum
				25% 50% 75% 100%
SCC *N*= 91	Any Mutation	50	54.9	
	PIK3CA	26	28.6	
	FBXW7	6	6.6	
	CTNNB1	8	8.8	
	PTEN	6	6.6	
	CDKN2A	5	5.5	
	NRAS	5	5.5	
	PPP2R1A	6	6.6	
	KRAS	2	2.2	
	HRAS	4	4.4	
	FGFR3	1	1.1	
AC *N*= 30	Any Mutation	10	33.3	
	PIK3CA	3	10.0	
	FBXW7	3	10.0	
	CTNNB1	0	0.0	
	PTEN	2	6.7	
	CDKN2A	2	6.7	
	NRAS	1	3.3	
	PPP2R1A	1	3.3	
	KRAS	2	6.7	
	HRAS	0	0.0	
	FGFR3	0	0.0	
ASC *N*= 16	Any Mutation	6	37.5	
	PIK3CA	4	25.0	
	FBXW7	0	0.0	
	CTNNB1	0	0.0	
	PTEN	0	0.0	
	CDKN2A	0	0.0	
	NRAS	1	6.3	
	PPP2R1A	0	0.0	
	KRAS	1	6.3	
	HRAS	1	6.3	
	FGFR3	0	0.0	

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