Genetic mutation analysis of the malignant transformation of sinonasal inverted papilloma by targeted amplicon sequencing

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Abstract

Background. The mechanism underlying the malignant transformation of inverted papilloma (IP) has not yet been elucidated.

Methods. To clarify the genes responsible for the malignant transformation, we analyzed 10 cases of IP, 8 of IP with dysplasia, and 11 of squamous cell carcinoma (SCC) by targeted amplicon sequencing.

Results. The number of mutant genes increased in the order of IP<dysplasia<SCC. Significant differences were observed in the mutation rates of 3 genes (*KRAS, APC* and *STK11*) in particular. *TP53* was altered frequently in each group and might be involved in malignant transformation based on to the site of the mutation. A comparison of the genetic variants by region of IP tissue among patients with IP alone, and those with dysplasia or SCC revealed significant differences in the mutation rate of the *KRAS* gene.

Conclusion. Identification of genetic mutations in *KRAS* is effective for predicting the malignant transformation of IP.

Keywords: inverted papilloma, malignant transformation, squamous cell carcinoma, targeted amplicon sequence, NGS

Introduction

Sinonasal papilloma accounts for 0.4-4.7% of all nasal tumors and is histopathologically classified into three types: inverted papilloma (IP), exophytic papilloma and oncocytic papilloma.[1] IP represents the most commonly diagnosed subtype of sinonasal papilloma with an incidence of 0.74-1.5 cases per 100.000 inhabitants per year.[2] IP is pathologically regarded as a benign tumor, but its malignant transformation is known to occur at a rate of 3.8-10%.[3-6] Therefore, the standard treatment for IP is complete resection. However, it is impossible to completely diagnose the malignant transformation of IP by macroscopic findings and computed tomography (CT) or magnetic response imaging (MRI) before surgical resection. Most of the malignant tissues present as squamous cell carcinoma (SCC). SCC within IP has been reported to occupy 10 to 95% of the total tumor volume.[7] Therefore, a preoperative biopsy may not include areas of the IP showing malignant change and, therefore, is not always effective in assisting the clinician in reaching a correct diagnosis. Therefore, multiple preoperative biopsies from different sites within the tumor tissue may be necessary to prevent misdiagnosis.

Recently, next-generation sequencer (NGS) has been widely used at the forefront of research, affording considerable progress in the identification of the responsible genes in several diseases.[8] The clinical applications of whole genome sequencing and whole exosome sequencing were limited in terms of the cost and time required; however, it has recently become possible to efficiently retrieve the target genes by amplicon sequencing.[9] These technological innovations mean that NGS can now be clinically applied for some types of tumors, including head and neck tumors.[10]

Although many investigations into the etiology and behavior of IP have been undertaken, the exact mechanism of its malignant transformation has not yet been fully elucidated. To clarify the genetic variants involved in the malignant transformation of IP, we analyzed the genetic variants in the tissues associated with IP, dysplasia and SCC by target amplicon sequencing with NGS and compared the differences in variants between the tissues. If the genes responsible for the malignant transformation of IP can be identified, it will become possible to predict malignant change in IP through examining alterations in those genes.

Materials and methods

Patient selection and histologic review

We selected 29 patients with IP or SCC involving the nasal or paranasal sinus who were treated at Hokkaido University Hospital between 2007 and 2015. Of the 29 patients, 10 had IP alone, 8 had IP with dysplasia, and 11 had SCC (Figure 1). Of the 11 cases with SCC, 5 had coexisting IP. In cases in which IP coexisted with dysplasia or SCC, the region of each different tissue was evaluated separately. Patients consisted of 21 males and 8 females, with a median age of 64 years (range 44-79 years). All hematoxylin & eosin-stained specimens were reviewed, and histopathological type was confirmed by 2 expert pathologists (S.T. and H.N.). This study was approved by the Ethics Committee of the Hokkaido University Hospital Clinical Research management unit, and informed consent was obtained from each patient.

DNA extraction

Formalin-fixed paraffin-embedded (FFPE) tissue samples were cut into 10 μm slices to create 5 specimens, and then genomic DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, NRW, Germany). The concentration of the genomic DNA was measured by Qubit dsDNA BR Assay

Kit (Invitorogen, Carlsbad, CA, USA) and GeneRead DNA QuantiMIXE Assay Kit (Qiagen).

Target amplicon sequencing

We used TruSight Tumor 26 (Illumina, San Diego, CA, USA) as an amplicon sequence panel. This panel can sequence 175 amplicon regions of 26 genes that are closely related to solid tumors of the lung, colon, melanoma, stomach, and ovary: *AKT1, EGFR, GNAS, NRAS, STK11, ALK, ERBB2, KIT, PDGFRA, TP53, APC, FBXW7, KRAS, PIK3CA, BRAF, GFR2, MAP2K1, PTEN, CDH1, FOXL2, MET, SMAD4, CTNNB1, GNAQ, MSH6,* and *SRC*.

Creation of a DNA library

Using the obtained genomic DNA, we then amplified the target genes by a polymerase chain reaction (PCR) method with Gene Read DNA seq Panel PCR Regent V2 (Qiagen). To collect the amplified DNA, the PCR products were added to Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA), bound to magnetic beads, and then eluted from the magnetic beads. Terminal repair and adapter ligation were performed using a Gene Read DNA Library Core Kit (Qiagen) to prepare a purified DNA library. The DNA library was amplified by PCR with a GeneRead DNA Amp Kit (Qiagen), and purified

using a MinElute PCR Purification Kit (Qiagen). The amplified DNA library was sequenced by Miseq (Illumina).

Data analysis

The FastQ file obtained from Miseq by target amplicon sequencing was analyzed using the BaseSpace Variant Studio Ver 2.2 analysis server provided by Illumina. We detected single nucleotide polymorphisms (SNPs) with a base insertion and deletion. The SNPs were limited to mutations causing amino acid substitutions with a positive mutation rate of more than 10%.

Statistical analysis

For statistical analysis, Excel statistics version 1.03 (Social Information Service, Tokyo, Tokyo, Japan) was used. Kruskal-Wallis test was used to compare the number of mutant genes among the 3 groups, and chi-square test was used to compare the mutation rate of each gene. A p value of less than 0.05 was judged to be statistically significant.

Results

Correlations between genetic variants and histological type

We could detect one or more genetic mutations in each of the 29 cases, with a

total of 129 mutations with amino acid substitutions detected. In the 26 genes analyzed using the sequence panel, the average number of mutant genes was 2.9 (range 1-5) in the IP tissue from patients with IP alone, 4.4 (range 3-6) in the dysplastic tissue from patients with IP with dysplasia, and 5.7 (range 3-9) in the carcinoma tissue from patients with SCC with or without IP (Figure 2). The number of mutant genes increased in the order of IP<dysplasia<SCC, and there was a significant difference in the number of mutated genes between the IP and SCC tissue ($P = 0.042$, Figure 3). When dysplasia was divided into two groups based on the grading of dysplasia, the average number of mutant genes was 4.7 (range 3-6) in the low-grade dysplastic tissues (case number 11, 12, 13, 14, 17 and 18) and 3.5 (range 3-4) in the high-grade dysplastic tissues (case number 15 and 16). There was no significant difference between the two groups in terms of the number or pattern of mutant genes. When SCC was further divided into two groups, the average number of mutant genes was 5.6 (range 4-7) in the carcinoma tissue from patients with SCC with IP and 5.8 (range 3-9) in the carcinoma tissue from patients with SCC without IP, which we regarded as de novo SCC. There was no significant difference between the two groups in terms of the number or

pattern of mutant genes.

Analysis of each gene revealed significant differences in the mutation rates of the following 3 genes among the IP, dysplasia and SCC groups (Table 1). *KRAS* and *APC* were found to be altered less frequently in the IP group (10 and 0%), while they were more frequently mutated in the dysplasia (75 and 50%) and SCC (73 and 64%) groups. No alterations in *STK11* were found in the IP and dysplasia groups, while it was more frequently mutated in the SCC group (36%). *TP53* was found to be altered frequently in each group (IP, 70%; dysplasia, 75%; SCC, 91%), with no significant differences observed among the three groups.

Variants detected multiple times across all patients

Table 2 shows the position on the chromosome and frequency of genetic variants detected multiple times across the 29 patients. Relatively frequent alterations were observed for *TP53,* g.7579472G>C (26%; overall, IP, 30%; dysplasia, 25%; SCC, 27%) and *APC*, g.112175770G>A (24%; overall, IP, 0%; dysplasia, 25%; SCC, 27%).

Differences in genetic variants in the same patient

We compared the differences in genetic variants between the regions of IP

and dysplastic tissue in the same patient with IP with coexistent dysplasia. A total 39 mutant genes were found in either the IP or dysplastic tissue in 8 cases, while 4 mutant genes (10%) were found in the IP alone, and 6 mutant genes (15%) in the dysplastic tissue alone (Figure 4). Similarly, we compared the differences in genetic variants between the IP and SCC regions in the same patient with IP with coexistent SCC. A total 29 mutant genes were found in either the IP or SCC tissue in 5 cases, while 1 mutant gene (3%) was found in the IP alone, and 2 mutant genes (7%) in the SCC tissue alone (Figure 5). In both IP with dysplasia and IP with SCC, little difference was observed in the genetic variants between the IP tissue and coexisting dysplasia or SCC tissue.

Genetic variants in the IP tissue among patients with IP alone, IP with dysplasia and IP with SCC

Furthermore, we compared genetic variants in the IP tissue among patients with IP alone, IP with dysplasia and IP with SCC (Figure 6). The number of mutant genes increased in the order of IP<IP with dysplasia<IP with SCC, and there was a significant difference in the number of mutated genes in the IP tissue between patients with IP alone and those with IP with SCC (P =

0.030, Figure 7). The analysis of each gene revealed that there was significant difference in the genetic mutation of *KRAS* in the IP tissue between patients with IP and those with IP with dysplasia/SCC (P = 0.005, Table 3). Therefore, we calculated the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for patients with coexistent dysplasia or SCC. Results showed that the sensitivity of the detection of dysplasia or SCC using the *KRAS* mutation was 85%, the specificity was 90%, the PPV was 91% and the NPV was 75%.

Discussion

Recently, several investigators have reported on candidate biomarkers for the malignant transformation of sinonasal IP. Cyclooxygenase-2, which is induced by mitogenic and inflammatory stimuli and promotes tumorigenesis, has been reported to be overexpressed significantly in IP with SCC compared to the level observed in benign IP.[11] Human papilloma virus (HPV) shows higher viral loads in patients with IP/SCC than in those with IP.[12] A recent systemic review with meta-analysis found a statistically significant association between HPV infection and malignant transformation of IP.[13]

Furthermore, proapoptotic factors such as p53, p21, p16, p27, tissue factor pathway inhibitor-2, p63, bcl-2 family, Ki-67, proliferating cell nuclear antigen and intercellular adhesion molecules have all been evaluated as possible contributors to IP malignant transformation.[14-19] However, there have been few reports on genetic variants associated with the malignant transformation of IP to date. Udager et al. reported that EGFR mutations were found in 88% of patients with IP and 77% of those with IP with SCC, but this difference was not significant.[20]

Therefore, we undertook a comprehensive analysis of genetic alterations in 26 genes closely related to solid tumors by target amplicon sequencing with NGS. As a result, the number of mutant genes was found to increase in the order of IP<dysplasia<SCC (Figure 3). In particular, there were significant differences in the mutation rates of 3 genes: *KRAS, APC,* and *STK11* (Table 1). Furthermore, there was a significant difference in the genetic mutation of *KRAS* in the IP tissue between patients with IP alone and those with IP with dysplasia/SCC (Table 3). This finding strongly suggested that *KRAS* mutations could be involved in the malignant transformation of IP. Regarding the genetic alterations in *KRAS*, Udager et al. reported that

KRAS mutations were identified in all cases with oncocytic papilloma, a subtype of sinonasal papilloma, and oncocytic papilloma-associated SCC.[21] However, they also reported that there were no *KRAS* mutations in either IP or IP-associated SCC.

On the other hand, *TP53* was found to be altered frequently in all groups (IP, 70%; dysplasia, 75%; SCC, 91%), but there were no significant differences among the three groups in this study (Table 1). However, in the analysis of gene mutation site, the g. 7570312C>A mutation was detected in 7% of all cases, with all cases limited to the SCC group (18%). In addition, the g. 7577150T>A mutation was detected in 10% of all cases, with all cases found in patients with either dysplasia or SCC (13% and 18%, respectively) (Table 2). Lin et al. showed that tissue from IP with carcinoma is positively stained for p53 at more than twice the frequency than that observed in tissue from IP alone based on an immunohistochemical approach (62% versus 30%), and suggested that alterations in p53 are important in the progression of IP to malignant disease.[16] These results suggest that p53 protein dysfunction, dependent on the site of the *TP53* mutation, could be involved in the malignant transformation of IP.

Currently, the diagnosis of SCC derived from IP requires the pathological existence of IP. However, if the IP is completely replaced by carcinoma, or if carcinoma alone occurs after complete IP resection, a differential diagnosis between SCC derived from IP and de novo SCC would be impossible. However, if the IP and SCC occur independently and the SCC invades the nearby IP, de novo SCC might be misdiagnosed as SCC derived from IP. In this study, we compared genetic variants between SCC with IP and de novo SCC, and no significant differences were observed between the two groups in terms of the number and pattern of mutant genes. Therefore, this study indicated that a differential diagnosis between SCC derived from IP and de novo SCC is impossible based on genetic alterations.

As IP occasionally progresses to malignant transformation, its standard treatment is complete resection. The nasal and paranasal sinuses, where IP arises, are anatomically adjacent to important organs such as the orbit and skull base; therefore, extended surgery for carcinoma derived from papilloma could have a great influence on postoperative complications and dysfunction. Accordingly, if we can predict the incidence of carcinoma within IP tissue prior to surgery, we could set an appropriate resection line and avoid

unnecessarily extensive surgery. However, it is currently impossible to completely diagnose the malignant transformation of IP by macroscopic findings and CT or MRI before surgical resection. Furthermore, even if a preoperative biopsy is performed, carcinoma tissue might not be contained in the biopsied specimen. Therefore, it would be clinically very beneficial to develop a new preoperative method for the diagnosis of the malignant transformation of IP.

We examined the differences in genetic variants between the IP and dysplasia or SCC tissues in the same patient. As a result, a total of 39 mutant genes were found in either the IP or dysplastic tissue, while 4 mutant genes (10%) were found in the IP tissues alone and 6 mutant genes (15%) were found in the dysplastic tissue alone (Figure 4). Similarly, a total of 29 mutant genes were found in either the IP or SCC tissue, while 1 mutant gene (3%) was found in the IP tissue alone and 2 mutant genes (7%) were found in the SCC tissue alone (Figure 5). Accordingly, there was little difference in the genetic variants between IP and the coexisting dysplasia or SCC. These results indicated that it might be possible to predict genetic variants in carcinoma within IP by genetic mutation analysis of the biopsied IP tissue.

Furthermore, we compared genetic variants in the IP tissue among patients with IP alone, IP with dysplasia and IP with SCC. As a result, significant differences were observed in the mutation rates of the *KRAS* gene in the IP tissue between patients with IP and those with IP with dysplasia/SCC (Table 3). Regarding other genes, no genetic mutations of *APC*, *NRAS* and *STK11* were found in patients with IP alone, while such mutations were frequently found in the IP tissue from patients with coexistent dysplasia or SCC. These results suggested that genetic variants of *KRAS*, *APC*, *NRAS* and *STK11* in preoperative biopsy tissue could be predictive of the existence of carcinoma within the IP tissues and the risk of future malignant transformation. If we can predict the existence of carcinoma within IP tissue prior to surgery, it may be possible to select the following treatment strategy: minimally invasive endoscopic sinus surgery for IP with low risk of malignant transformation, while external approach or advanced endoscopic sinus surgery including the bone resection of orbit or skull base for IP with high risk of malignant transformation.

One limitation to this study is that only a limited number of cases were analyzed; therefore, it is necessary to pursue further analysis of a

greater number of cases. Further research will allow for the identification of mutant gene combinations and their mutation sites that can predict malignant transformation of IP more accurately and efficiently.

In conclusion, the results of this study suggested that genetic variants of *KRAS, APC* and *STK11* are involved in the malignant transformation of IP, and *KRAS*, in particular, is the key gene in predicting the malignant transformation of IP.

Conflict of Interest

No author has any conflict of interest.

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Figure legends

Figure 1. Histopathology of inverted papilloma, dysplasia and squamous cell carcinoma

(A) inverted papilloma (IP), (B) dysplasia with IP, (C) squamous cell carcinoma.

Figure 2. Mapping of genetic variants among patients with inverted papilloma, dysplasia and squamous cell carcinoma

Genetic variants were compared among patients with inverted papilloma (IP), dysplasia and squamous cell carcinoma (SCC). SCC was subdivided into SCC with IP and de novo SCC. Black: single nucleotide polymorphism, dark gray: insertion of nucleotide, light gray: deletion of nucleotide.

Figure 3. Mutated genes among patients with inverted papilloma, dysplasia and squamous cell carcinoma

A number of mutant genes among patients with inverted papilloma (IP), dysplasia and squamous cell carcinoma (SCC) were compared using Kruskal-Wallis test.

Figure 4. Mapping of genetic variants in inverted papilloma with dysplastic tissue

Genetic variants were compared between the inverted papilloma (IP) tissue and dysplastic tissue (Dys) in the same patient with IP with dysplasia. Black: single nucleotide polymorphism, dark gray: insertion of nucleotide, light gray: deletion of nucleotide.

Figure 5. Mapping of genetic variants in inverted papilloma with squamous cell carcinoma tissue

Genetic variants were compared between the inverted papilloma (IP) tissue and squamous cell carcinoma (SCC) tissue in the same patient with IP with SCC. Black: single nucleotide polymorphism, dark gray: insertion of nucleotide, light gray: deletion of nucleotide.

Figure 6. Mapping of genetic variants in the inverted papilloma tissue among patients with inverted papilloma alone, and those with dysplasia or squamous cell carcinoma

Genetic variants in the inverted papilloma (IP) tissue were compared among patients with IP alone, IP with dysplasia and IP with squamous cell carcinoma. Black: single nucleotide polymorphism, dark gray: insertion of nucleotide, light gray: deletion of nucleotide.

Figure 7. Mutated genes in the inverted papilloma tissue among patients with inverted papilloma alone, and those with dysplasia or squamous cell carcinoma

A number of mutated genes in the inverted papilloma tissue among patients with inverted papilloma (IP) alone, and those with dysplasia or squamous cell carcinoma (SCC) were compared using Kruskal-Wallis test.

Figure 1

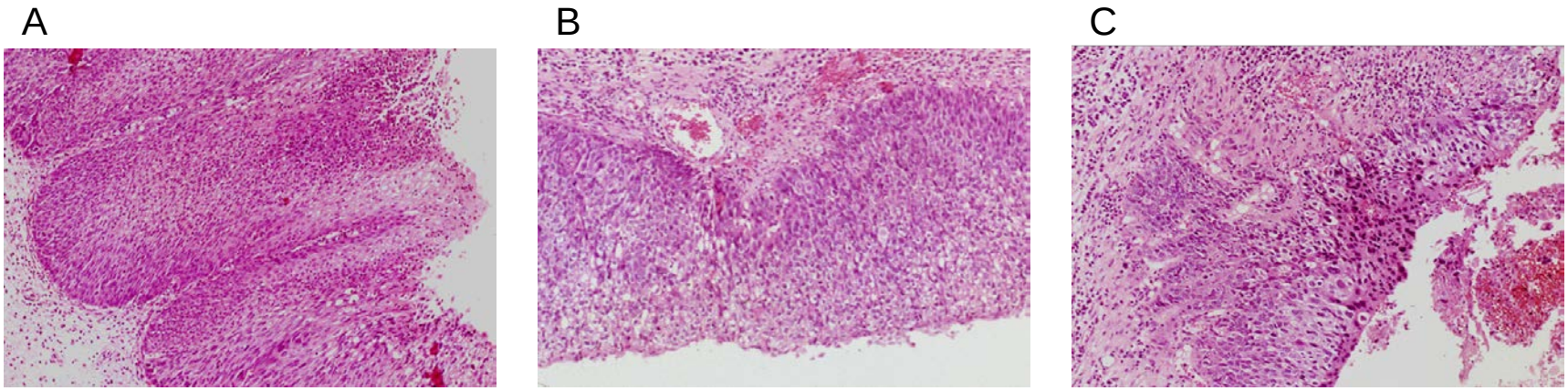

Figure 2

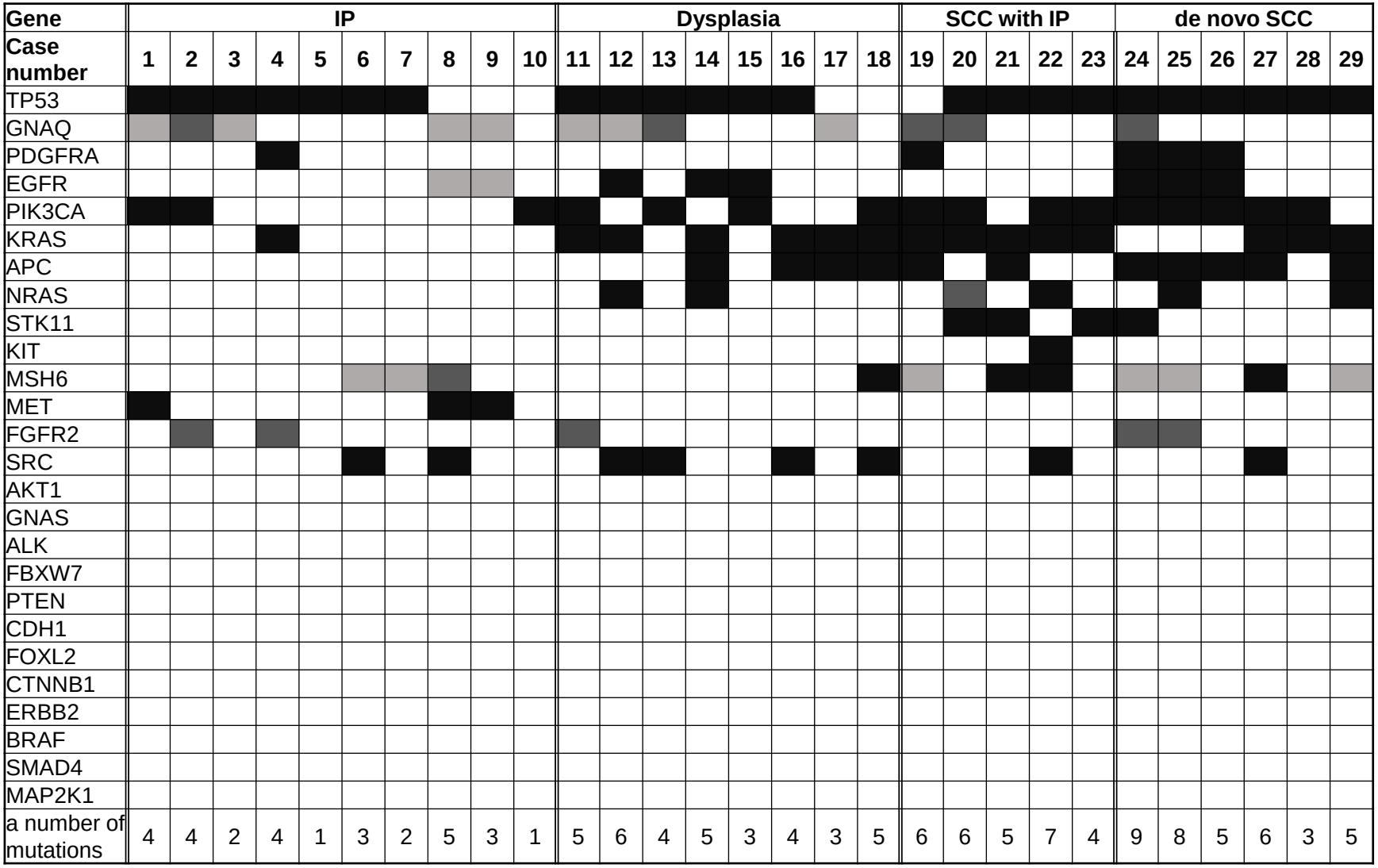
Gene
IP
Dysplasia
SCC with IP
de novo SCC
Case number
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
TP53
GNAQ
PDGFRA
EGFR
PIK3CA
KRAS
APC
NRAS
STK11
KIT
MSH6
MET
FGFR2
SRC
AKT1
GNAS
ALK
FBXW7
PTEN
CDH1
FOXL2
CTNNB1
ERBB2
BRAF
SMAD4
MAP2K1
a number of mutations
4 4 2 4 1 3 2 5 3 1 5 6 4 5 3 4 3 5 6 6 5 7 4 9 8 5 6 3 5

Figure 3

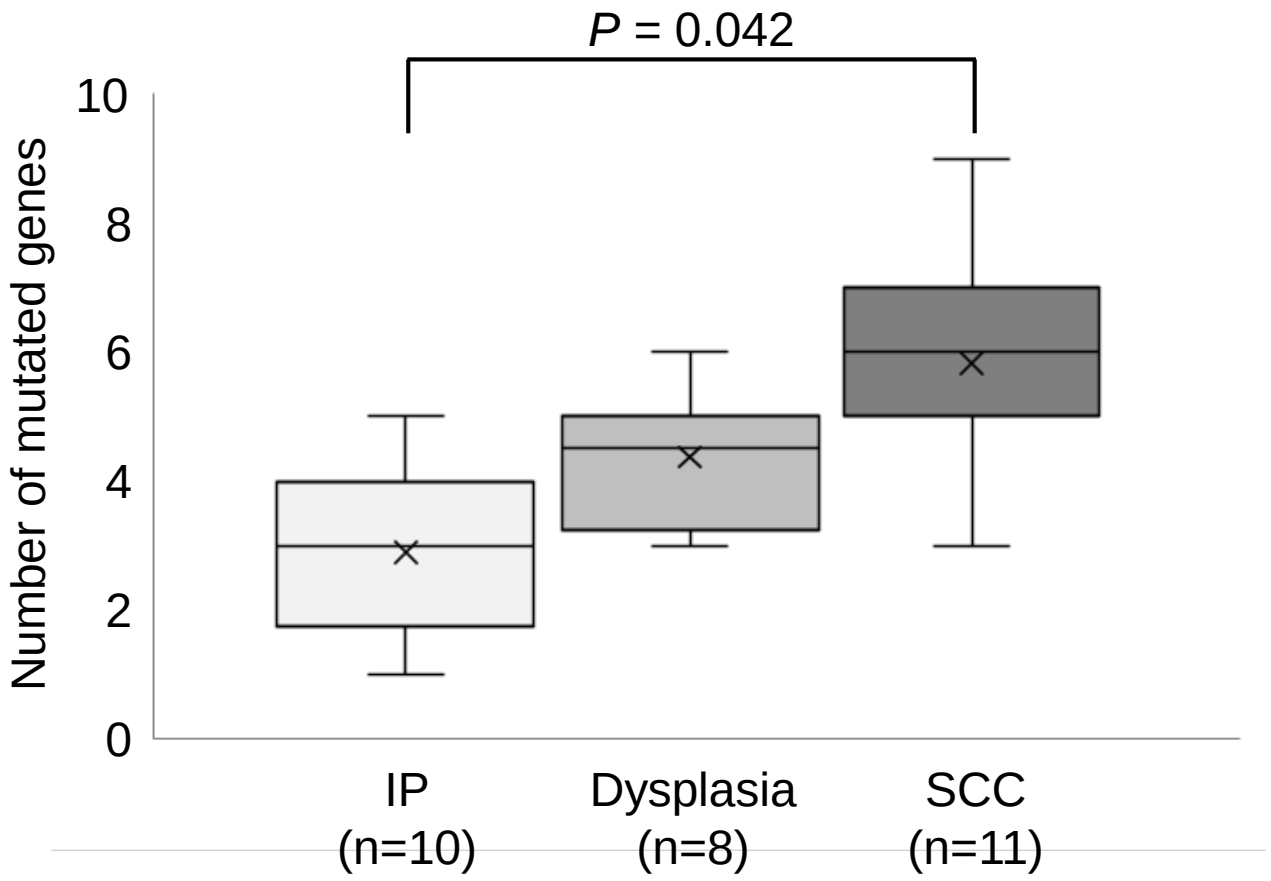
P = 0.042
Number of mutated genes
10
8
6
4
2
0
IP
(n=10)
Dysplasia
(n=8)
SCC
(n=11)

Figure 4

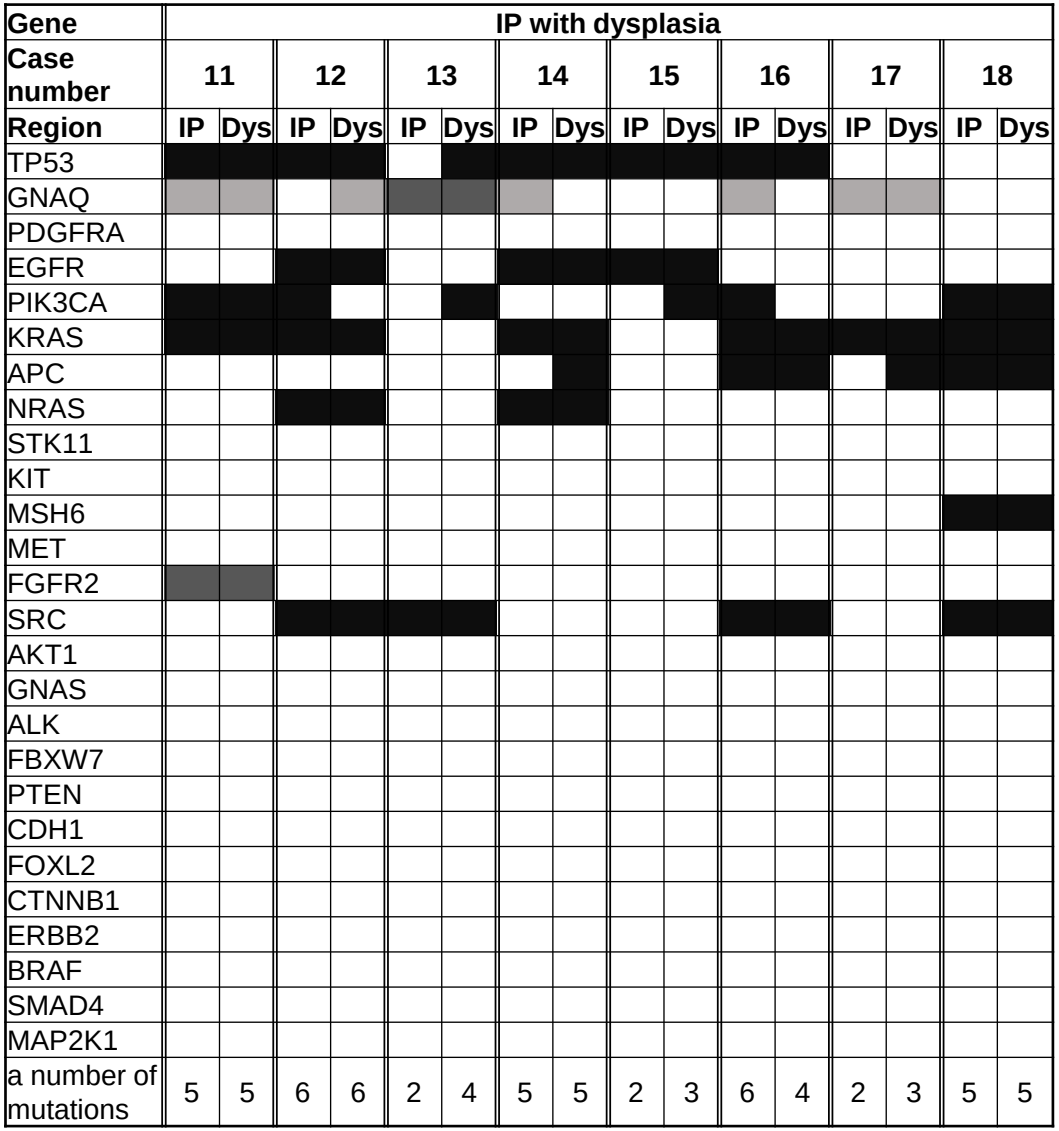
Gene
IP with dysplasia
Case number
11
12
13
14
15
16
17
18
Region
IP
Dys
TP53
GNAQ
PDGFRA
EGFR
PIK3CA
KRAS
APC
NRAS
STK11
KIT
MSH6
MET
FGFR2
SRC
AKT1
GNAS
ALK
FBXW7
PTEN
CDH1
FOXL2
CTNNB1
ERBB2
BRAF
SMAD4
MAP2K1
a number of mutations
5 5 6 6 2 4 5 5 2 3 6 4 2 3 5 5

Figure 5

Gene	IP with SCC									
Case number	19		20		21		22		23	
Region	IP	SCC	IP	SCC	IP	SCC	IP	SCC	IP	SCC
TP53										
GNAQ										
PDGFRA										
EGFR										
PIK3CA										
KRAS										
APC										
NRAS										
STK11										
KIT										
MSH6										
MET										
FGFR2										
SRC										
AKT1										
GNAS										
ALK										
FBXW7										
PTEN										
CDH1										
FOXL2										
CTNNB1										
ERBB2										
BRAF										
SMAD4										
MAP2K1										
a number of mutations	5	6	5	6	4	5	8	7	4	4

Figure 6

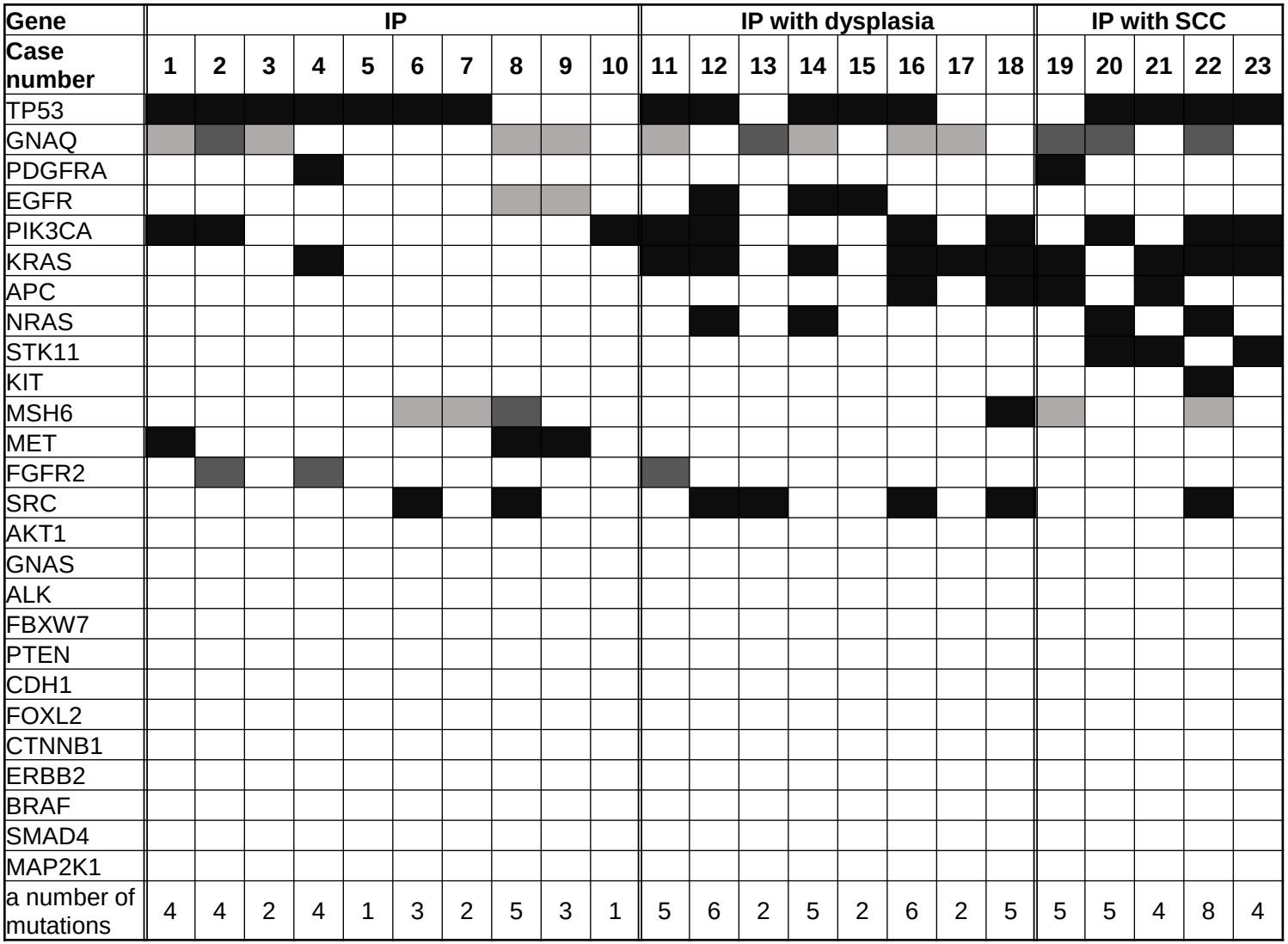
Gene
IP
IP with dysplasia
IP with SCC
Case number
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
TP53
GNAQ
PDGFRA
EGFR
PIK3CA
KRAS
APC
NRAS
STK11
KIT
MSH6
MET
FGFR2
SRC
AKT1
GNAS
ALK
FBXW7
PTEN
CDH1
FOXL2
CTNNB1
ERBB2
BRAF
SMAD4
MAP2K1
a number of mutations
4 4 2 4 1 3 2 5 3 1 5 6 2 5 2 6 2 5 5 5 4 8 4

Figure 7

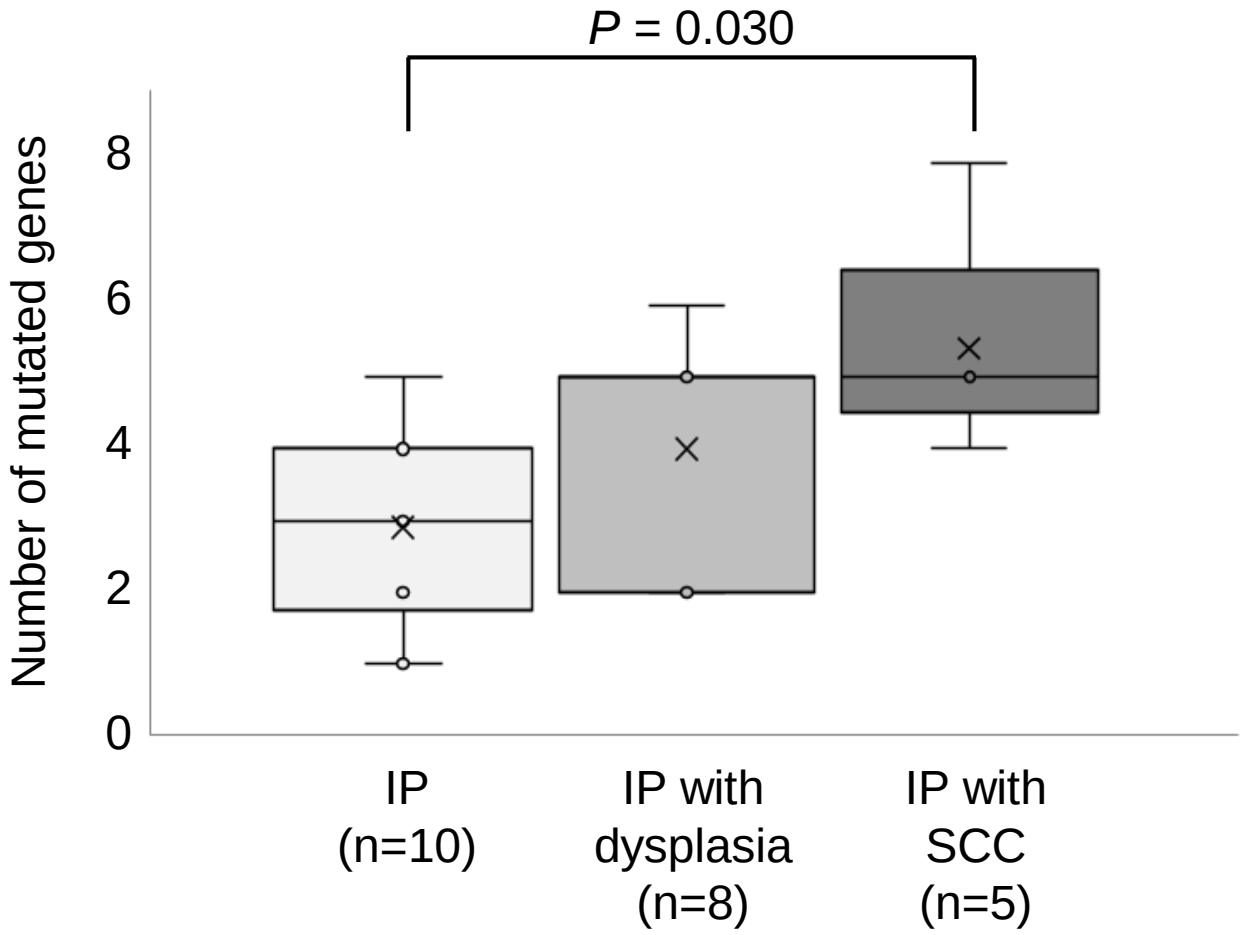
P = 0.030
Number of mutated genes
0
2
4
6
8
IP
(n=10)
IP with
dysplasia
(n=8)
IP with
SCC
(n=5)

Table 1. Mutation rate in inverted papilloma, dysplasia and squamous cell carcinoma

Gene	Mutation rate (%)				*P*
	All	IP	Dysplasia	SCC	
TP53	79	70	75	91	0.467
GNAQ	41	50	50	27	0.483
PDGFRA	17	10		36	0.088
EGFR	28	20	38	27	0.711
PIK3CA	55	30	50	82	0.054
KRAS	52	10	75	73	**0.004**
APC	38		50	64	**0.008**
NRAS	21		25	36	0.113
STK11	14			36	**0.022**
KIT	3			9	0.428
MSH6	38	30	13	64	0.062
MET	10	30			**0.041**
FGFR2	17	20	13	18	0.911
SRC	28	20	50	18	0.248

IP: inverted papilloma, SCC: squamous cell carcinoma

Table 2. Variants detected multiple times across all cases

Gene	Mutation	Position on chromosome	Mutation rate (%)			
			All	IP	Dysplasia	SCC
TP53	C>A	7570312	7			18
	T>A	7577150	10		13	18
	G>T	7578511	10	10		18
	G>C	7579472	26	30	25	27
GNAQ	G>GAA	80343587	14	10	13	18
	G>GA	80343587	17	20	13	18
	GAA>G	80343587	7	10		9
	GA>G	80343587	17	10	13	9
PDGFRA	A>G	55141055	7	10		9
	C>T	55152040	7		13	9
EGFR	C>CGGG	55249012	14	10	13	18
	AA>TT	55249013	10		25	9
	C>CGGGTTT	55249012	7	10	13	0
	G>A	55249063	7	10		9
PIK3CA	C>T	178917627	7	10		9
KRAS	G>A	25378661	14	10	25	9
	A>G	25362777	10	10	25	0
APC	C>T	112174550	7		13	9
	G>A	112175642	10			27
	G>A	112175770	24		25	27
STK11	A>G	153247353	7			18
MSH6	A>T	48030559	10	20		9
	AC>A	48030639	10	10	13	9
	A>AC	48030639	10	10	13	9
	T>A	48030692	17	10	13	27

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Table 3. Mutation rate in the inverted papilloma tissue among patients with inverted papilloma alone, and those with dysplasia or squamous cell carcinoma

Gene	Mutation rate (%)			*P*
	All	IP	Dysplasia/SCC	
TP53	70	70	69	1.000
GNAQ	57	50	62	0.897
PDGFRA	9	10	8	1.000
EGFR	22	20	23	1.000
PIK3CA	43	30	54	0.4719
KRAS	48	10	77	**0.005**
APC	17		31	0.169
NRAS	17		31	0.169
STK11	13		23	0.315
KIT	4		8	1.000
MSH6	26	30	23	1.000
MET	13	30		0.135
FGFR2	13	20	8	0.807
SRC	30	20	38	0.619

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