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Mutation Profiling Indicates High Grade Prostatic Intraepithelial Neoplasia as Distant Precursors of Adjacent Invasive Prostatic Adenocarcinoma

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Abstract

INTRODUCTION—High Grade Prostatic Intraepithelial Neoplasia (HGPIN) is the putative precursor lesion to prostatic adenocarcinoma (PCa), but the precise relationship between HGPIN and PCa remains unclear.

METHODS—We performed a molecular case study in which we studied mutation profiles of six tumor-associated HGPIN lesions in a single case of *TMPRSS2:ERG* fusion positive Gleason score 7 PCa that we had previously mapped for somatic mutations in adjacent Gleason pattern 3 and 4 foci, using microdissection and targeted deep-sequencing.

RESULTS—A total of 32 tumor-specific mutated sites were successfully amplified and sequenced, including 25 truncal mutations and 7 mutations that were specific to either the Gp3 or Gp4 foci. All six HGPIN foci shared the same tumor-specific *TMPRSS2:ERG* fusion breakpoint, establishing that they were all clonally related to the adjacent invasive tumor. Among the 32 gene targets mutated in the invasive tumor, only mutation of the *OR2AP1* gene, a truncal mutation, was found in a single focus of HGPIN. The remaining gene targets that were successfully sequenced were wild-type in all other HGPIN foci.

DISCUSSION—This study demonstrates the feasibility of targeted mutation profiling of HGPIN lesions, which will be important to understand PCa tumorigenesis. The results in this case, showing a remarkable absence of truncal mutations in HGPIN lesions bearing the tumor-specific *ERG* fusion, indicate HGPIN lesions may be relatively stable genetically and argue against a stepwise clonal evolution model of HGPIN to PCa.

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INTRODUCTION

Prostate cancer (PCa) is the most common malignancy of visceral organs in men, and it is estimated that PCa will kill 26,120 men in the United States in 2016 (1). High Grade Prostatic Intraepithelial Neoplasia (HGPIN) is a putative precursor lesion to PCa (2) (3) (4). Earlier studies have demonstrated that HGPIN shares some molecular features of PCa. Similar to the invasive PCa, HGPIN can show 8p12-21 allelic loss (5) and gain of chromosomes 7, 8, 10, and 12 (6) (7). *TMPRSS2:ERG* fusions, which occur in about half of PCa, were detected in 10–20% of HGPIN (8–10). HGPIN may also show increased protein expression of c-MYC (11), c-ERBB-2 (HER-2) (12), c-MET (13), BCL-2 (14), and alpha-methylacyl-CoA racemase (15), as well as mutant p53 (16). Different from invasive PCa, HGPIN generally does not harbor *PTEN* deletion (17) (18) or *RB* loss (16), although conflicting data have been published (19) (20). Based on these data, it is not yet clear whether HGPIN is an immediate precursor of PCa, and genetic alterations that may drive HGPIN to invasive PCa remain to be established.

The precise relationship between Gleason pattern 3 (Gp3) and Gleason pattern 4 (Gp4) tumors, and whether Gp3 progresses to Gp4, has similarly been uncertain. Studies from our laboratory and others have used *TMPRSS2:ERG* fusion breakpoint sequencing to demonstrate a clonal relationship between adjacent Gp3 and Gp4 tumor foci in Gleason score 7 (GS7) PCa (21, 22). Since then, by whole exome sequencing, we have confirmed that these clonal Gp3 and Gp4 tumor foci share multiple mutations (truncal mutations), but that many additional mutations may be unique to the clonally related Gp3 and Gp4 tumors (Sowalsky et al., submitted for publication). In this study, we have isolated multiple HGPIN lesions associated with a GS7 tumor, in conjunction with adjacent Gp3 and Gp4 foci, in order to determine the relationship between these entities. Our results establish that the HGPIN lesions, Gp3, and Gp4 foci all share a common origin, but multiple mutations common to the Gp3 and Gp4 foci were not detected in the HGPIN lesions. These findings argue against a straightforward stepwise progression from HGPIN to PCa, and indicate that HGPIN may be genetically stable entities and represent distant precursors to PCa.

MATERIALS AND METHODS

Tissue Selection

Tissue from radical prostatectomy (RP) specimens was collected and deidentified in accordance with Beth Israel Deaconess Medical Center IRB protocol #2010-P-000254. Invasive tumors were graded using the modified Gleason grading system established by the 2014 International Society of Urological Pathology (ISUP) consensus conference (23, 24). RP cases of GS7 PCa were reviewed for tumor-associated HGPIN lesions. A single case harboring multiple foci of HGPIN was selected. The patient was a 66-year-old white male with PCa of ISUP Grade Group 2 (Gleason score 3+4=7) staged pT2cN0 (patient 2 in our previous study (21)). The entire prostate was submitted for histological examination. Prostatic tissue was fixed in formalin, processed, and embedded in paraffin using standard methods. A total of six ERG-positive tumor-associated HGPIN foci (concordant with the ERG-positive PCa) were identified. Genetic alterations in the adjacent Gp3 and Gp4 tumors had been previously mapped using whole exome sequencing (Sowalsky et al., submitted for

publication). Gp3, Gp4 and HGPIN lesions were identified by a urological pathologist (HY). HGPIN lesions were diagnosed based on architecturally benign glands lined by cytologically malignant luminal cells with visible nucleoli at 200× and presence of basal cells at the periphery, after excluding intraductal carcinoma (25). Tumor-associated HGPIN was defined as HGPIN located either within the index tumor or on the edge of the index tumor. Six-micron sections were stained with H&E, anti-ERG (clone EPR3863, Epitomics, Burlingame, CA), and PIN-4 antibodies (Biocare Medical, Concord, CA) according to manufacturer's suggested protocols.

Laser-Capture Microdissection

Six-micron serial sections from this case were cut onto polyethylene naphthalate metal frame slides (Applied Biosystems, Foster City, CA) and lightly stained with the Arcturus Paradise Plus Staining Kit (Applied Biosystems, Foster City, CA). Using consecutive ERG and PIN-4 immunostains as references, epithelial cells were collected using laser-capture microdissection from a total of 9 samples, including 6 tumor-associated HGPIN foci (A to F), 1 normal, 1 Gp3, and 1 Gp4 tumor focus. Epithelial cells from the 9 samples were captured onto separate caps, using 20-micron infrared pulses and excised from the adjacent tissue using the ultraviolet laser on an ArcturusXT Nikon Eclipse Ti-E microdissection system. Each captured region was checked against the reference slides for accuracy prior to DNA extraction.

Library Preparation and Deep Sequencing

Genomic DNA (gDNA) was extracted and isolated using the QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA). Multiplex PCR was performed to amplify 46 tumor mutation-specific targets from no more than 10ng of gDNA per sample. After this initial PCR amplification, the products were purified and used as templates for multiple secondary amplifications using single primer pairs. This resulted in successful amplification of 32 targets in one or more of the HGPIN lesions, including 7 grade-specific (found in only the Gp3 or Gp4 focus) and 25 Gp3 and Gp4-shared mutations identified in the adjacent invasive tumor. For each HGPIN, normal, and tumor control samples, a DNA library was prepared by pooling 5 ng of each PCR product per target. The libraries were then fragmented and prepared for NGS using NEBNext Library Prep for Illumina (New England BioLabs, Ipswich, MA), clustered, and sequenced on a MiSeq (Illumina, San Diego, CA) with 100 cycles paired end (100×100) and 7 additional indexing cycles.

Original sequence data has been deposited in the NCBI Sequence Read Archive under Accession ID SRP074297.

Data Alignment and Determination of Mutations

The sequencing data files were generated from the image data, demultiplexed on CASAVA 1.8.2, and subsequently downloaded in FASTQ format. FASTQ files were aligned to human genome (Hg19) using the Burrows-Wheeler Aligner, and mutations were identified using VarScan 2 for somatic analysis, comparing each cancer or HGPIN BAM file to the normal sequence data, and applying Fisher's exact test to determine whether a mutation was genuine or background. We then applied a filter that limited the sequencing data to only the regions

amplified by multiplex PCR, and performed a functional prediction with SNPEff (26) to give the amino acid changes for any known protein-coding regions.

Statistical Analysis

Assuming the minimal purity of HGPIN that was microdissected was 10%, and assuming normal ploidy, our technical lower limit to call a mutation was limited to read depth of at least 20 reads with one read called as mutant. However, with a false-positive probability (α) of 0.05 and a false negative probability (β) of 0.05, our ability to call definitely the genotype at any site was limited to those sites with read depth greater than or equal to 33 reads. This power analysis was performed using G*Power version 3 for Mac.

Quantitative PCR (qPCR)

SYBR Green-based qPCR for a relative quantification (C_T method) was performed on the positive Gp3 and Gp4 controls, negative controls, normal tissue, and HGPIN lesions. The template amounts for the Gp3, Gp4, and normal were 10 ng each. The template amount for the HGPIN lesions was <10 ng (too low to be accurately quantified). Each sample was performed in triplicate using patient-specific *TMPRSS2:ERG* fusion primers, with an *ERG* primer set for the internal reference *ERG* gene that was not associated with the *TMPRSS2:ERG* fusion. Additionally, three (10, 1, and 0.1 ng) template amounts of the Gp3 positive control DNA were used for the linearity analysis. 5 μ L of 2 $\times$ QuantiFast SYBR Green PCR master mix (Qiagen, Valencia, CA) was mixed with the forward and reverse primers each at a final concentration of 1 μ M and the respective DNA amounts to bring each sample to a final volume of 10 μ L. Cycling conditions specified by the manufacturer were used, for a total of 45 cycles using a StepOnePlus Real-Time PCR instrument (Life Technologies, Foster City, CA). Melting curve analysis was used to confirm primer pair specificity in that each well contained only a single amplified product.

RESULTS

Identification and Isolation of Tumor-associated HGPIN Lesions

TMPRSS2:ERG rearrangement is known to be an early genetic event in PCa development. Therefore, HGPIN lesions that showed concordant ERG staining patterns with the adjacent tumors were considered as potential precursors for invasive tumors. We reviewed H&E stains, PIN-4 and ERG immunostains of the selected RP case. A total of six spatially separated tumor-associated ERG-positive HGPIN lesions, HGPIN foci A to F, were identified. Figure 1 illustrates the relationship of HGPIN lesions with the invasive tumor. Figure 2 shows the concordant ERG immunoreactivity in the HGPIN foci A, C, and F, Gp3, and Gp4 tumors, indicating that they potentially share the clonal lineage. Figure 3 shows HGPIN-C and HGPIN-F at a higher magnification. Pictures of the remaining four foci (HGPIN-A, -B, -D, -E) at high magnifications are shown in Supplementary Figures 1 and 2. Epithelial cells of all the six HGPIN lesions were captured individually using laser-capture microdissection (LCM). The insert in Figure 3F shows the captured HGPIN-F on the LCM polymer cap, demonstrating a clean harvest with minimal contamination by surrounding tissue.

HGPIN Shares the Same *TMPRSS2:ERG* Breakpoints as Invasive PCa

Next, we asked if the six ERG-positive HGPIN lesions shared the same *TMPRSS2:ERG* breakpoints with the adjacent invasive tumor. Using patient-specific primers for the *TMPRSS2:ERG* breakpoints (21), we generated PCR products of ~150 bp from gDNA of all six foci of HGPIN, but not in the no-template negative control DNA (Figure 4A). The presence of a ~150 bp PCR product in each lane suggested that all six HGPIN lesions shared the same *TMPRSS2:ERG* fusion as the invasive tumor. Sanger sequencing of PCR products demonstrated that the breakpoints in all six foci were the same as the breakpoints of invasive tumor (Figure 4B). The breakpoint coordinate for *ERG* was chr21:39870028 and the *TMPRSS2* coordinate was chr21:42871313.

To rule out the possibility that detection of the fusion breakpoint in the HGPIN lesions was due to trace contamination from the adjacent tumor cells during LCM, we asked if the *TMPRSS2:ERG* fusion gene detected in the HGPIN lesions was present at comparable levels to the adjacent Gp3 and Gp4 invasive tumors. For this purpose, quantitative PCR (qPCR) was performed on the DNA extracted from Gp3, Gp4, normal tissue, and HGPIN samples. For this analysis, we set purity of our Gp3 sample to 1 because the microscopic anatomy of Gp3 is similar to that of HGPIN, i.e. well-defined glands surrounded by fibromuscular stroma. As shown in Figure 5A, relative quantity (RQ) values of Gp4, normal tissue, and HGPIN-F were 1.573, 0.030, and 0.492, respectively. The latter approximately 50% purity of fusion-positive cells in the HGPIN samples relative to the Gp3 sample was consistent with basal cells and residual normal luminal epithelial cells in the microdissected HGPIN, and confirms that the DNA containing the fusion breakpoint was not derived from trace contaminating tumor cells. The cribriform Gp4 samples had a higher purity compared to Gp3 samples, which is consistent with less stroma in the microdissected cribriform Gp4. Therefore, the results of purity analysis of HGPIN, Gp3, and Gp4 samples can be explained by their respective histological features. Taken together, these findings establish that the HGPIN foci were clonally related to the invasive tumor, as they all shared the same *TMPRSS2:ERG* breakpoints as the invasive Gp3 and Gp4.

OR2AP1 Truncal Mutation Detected in One HGPIN Lesion

We had previously carried out whole exome sequencing on the LCM purified adjacent Gp3 and Gp4 foci from this patient, as well as adjacent nonmalignant prostate, to identify a series of mutations that were common to both the Gp3 and Gp4 tumors (presumably truncal mutations), as well as mutations that were unique to the Gp3 or Gp4 foci (Sowalsky et al., submitted for publication). Therefore, we next tested whether the HGPIN lesions harbored any of the somatic mutations detected in the Gp3 and Gp4 tumors. We used PCR and amplicon resequencing to assess for the presence of 25 mutations that were shared by Gp3 and Gp4 tumors (truncal mutations), 3 mutations found only in the Gp3 focus, and 4 mutations found only in the Gp4 focus (primer sequences listed in Supplementary Table 1). As shown in Table 1, all six foci of HGPIN successfully generated mutational landscape data, with most of the 32 amplicons generating sufficient read depth to call a mutation with a false positive probability (α) = 0.05 and call a wild-type allele with a false negative probability (β) = 0.05.

Surprisingly, there were no mutations detected in the DNA from HGPIN lesions A, B, C, D, or E. In HGPIN-F, we detected only one mutation, which was in *OR2AP1* (a truncal mutation found in both the Gp3 and 4 foci that resulted in an amino acid change from leucine to phenylalanine at codon 283). The *OR2AP1* gene in HGPIN-F had a total coverage of 159 reads, with 44 reads (28%) for the missense thymine mutation (g.chr12:55969047G>T) (21 positive strand and 23 negative strand) and 113 reads (71%) for the normal coding guanine allele (54+, 59–) (Figure 5B; Table 1). Assuming normal ploidy and a heterozygous mutation, this 28% frequency is in excellent agreement with the estimated 50% purity of the HGPIN sample, and suggests that it is present in virtually all of the HGPIN-F cells.

DISCUSSION

The results of this case study show that the HGPIN lesions and adjacent Gp3 and Gp4 tumor foci in this patient had identical *TMPRSS2:ERG* breakpoints. Importantly, using qPCR for relative quantification of the *TMPRSS2:ERG* breakpoints, we confirmed that their detection in the HGPIN lesions was not caused by tissue contamination during LCM. Moreover, the absence of truncal mutations (found in the Gp3 and Gp4 tumors) in the HGPIN lesions excluded the possibility of a retrograde acinar colonization that may morphologically mimic HGPIN (27). Together these results establish that the HGPIN, Gp3 and Gp4 foci in this patient were clonally related, and support previous studies indicating that HGPIN is a precursor to PCa.

To further address the relationship between these clonal HGPIN lesions and adjacent Gp3 and Gp4 tumor foci, we assessed the HGPIN for somatic mutations found in the Gp3 and Gp4 tumor foci. We discovered that one of six HGPINs harbored a mutation in *OR2AP1*, a somatic mutation that was shared by the invasive Gp3 and Gp4 (but not present in nonmalignant prostate from this patient). *OR2AP1* codes for olfactory receptor family 2 subfamily AP member 1, a prostate-specific G-coupled receptor. Another member of the olfactory receptor family, prostate specific G protein-coupled receptor (PSGR), has been reported to be involved in development and progression of PCa (28) (29). However, the functional role of this *OR2AP1* mutation in PCa development is unclear. Therefore, although it may be involved in the progression of HGPIN to PCa, it may instead have emerged as a passenger mutation at some point after the *TMPRSS2:ERG* fusion.

Significantly, with the exception of this *OR2AP1* mutation in one HGPIN focus, we did not detect any of the other 32 mutations that we had observed in the adjacent invasive tumor. Of these, 25 were shared between the associated Gp3 and Gp4 tumors, so many or most are likely to reflect truncal mutations in the invasive tumor. Therefore, although the single *OR2AP1* mutation is consistent with a stepwise progression from HGPIN to invasive cancer, one would then expect to detect further intermediates containing additional truncal mutations. While it is possible that these were present but missed, we instead suggest that these HGPIN lesions persisted as a relatively genetically stable entity that was only distantly related to the adjacent Gp3 and Gp4 tumor foci. The lack of intermediates with additional truncal mutations may then reflect microheterogeneity within the HGPIN, with PCa in this case arising from a single HGPIN cell from a minor HGPIN subclone that already contained

multiple mutations. Alternatively, it is possible that a genomic alteration occurring in a HGPIN subclone with the *OR2AP1* mutation caused genomic instability and rapid accumulation of mutations leading to invasive cancer. Interestingly, we did detect an *MSH6* mutation in the Gp3 tumor from this case, which is often associated with further genomic instability. However, it was not found in the Gp4 focus, so is presumably not truncal (see Table 1).

Given the technical challenge of laser capture microdissection and subsequent molecular analysis of very small amounts of DNA from multiple HGPIN foci from the formalin-fixed paraffin-embedded tissue, our study has some limitations. In particular, the amount of gDNA recovered from each focus was variable, which may have contributed to different amplification efficiencies for each target. Consequently, not all targets generated sequencing results with comparable coverage in all samples. For example, HGPIN foci A, B, D, and E generated minimal or no reads for *OR2AP1*, therefore, we lack the statistical power for this amplicon to determine conclusively that we did not miss the *OR2AP1* mutation in these HGPIN lesions, and thus we cannot rule out the possibility that the *OR2AP1* mutation emerged in multiple HGPIN foci prior to the emergence of cancer. In addition, very low input DNA sequenced may be associated with unequal coverage, amplification artifacts, and contamination issues. Nonetheless, more targets were successfully sequenced than unsequenced in all samples, with a majority of targets having unbiased sequence coverage of greater than 50x, supporting our conclusions of a mutation-poor landscape in HGPIN lesions in this case.

Significantly, while our study was carried out in a single *TMPRSS2:ERG* fusion positive case, a very recent study examined genetic profiles of HGPIN and PCa in 6 fusion-negative Asian patients (30). Consistent with our current study, the HGPIN and PCa were found to be clonal based on shared genetic alterations in 4 cases. Moreover, significantly fewer mutations were found in the HGPIN versus PCa, indicating substantial genetic evolution from HGPIN to PCa with is consistent with our findings. Overall, while the data clearly establish a clonal relationship between HGPIN and PCa, they further indicate that HGPIN are relatively genetically stable and support clinical observations and the conservative management of men with HGPIN. However, given the limited applicability from this single case study, further studies are needed to elucidate mechanisms that may drive HGPIN to PCa, and to determine whether there are molecular features of some HGPIN that are associated with increased risk of progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Beth Israel Deaconess Medical Center Molecular Medicine core facility. Paired-end sequencing libraries were quantified, clustered, and sequenced at the Harvard Medical School Biopolymers Facility. The authors have no conflicts of interest to declare.

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Figure 1.

The topographic relationship between HGPIN lesions and invasive tumor. Six spatially separated HGPIN lesions (A–F) are present in the periphery of the Gleason score 7 tumor, located in the left posterior lobe in slices #4 and #5 (total 8 slices from apex to base).

Figure 2.

Gp3 component and three adjacent HGPIN lesions (black arrow = HGPIN-C; gray arrow = HGPIN-F; white arrow = HGPIN-A) (A–C, 40×) and Gp4 component on the same block (D–F, 100×): A and D, H&E; B and E, PIN-4 immunostain; C and F, ERG immunostain.

Figure 3.
HGPIN-C and HGPIN-F (200×): Left panel: HGPIN-C; Right panel: HGPIN-F. A and D, H&E; B and E, PIN-4 immunostain; C and F, ERG immunostain; Inset: dissected HGPIN-F on LCM Polymer cap.

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Figure 5.
 (A) The relative abundance of the *TMPRSS2:ERG* fusion in each sample by qPCR. (B)
 Identification of the *OR2AP1* trunk mutation in HGPIN-F using IGV.

Table 1

Sequencing Coverage of 32 Targets

Gene Target	Mutation	PIN-A Reads	PIN-B Reads	PIN-C Reads	PIN-D Reads	PIN-E Reads	PIN-F Reads	Normal Reads	Gp3 Reads	Gp4 Reads
Shared:										
<i>ACOXL</i>	M178I	6,588	7,884	281	147	157	20*	517	2*	75
<i>ARMC2</i>	A292G	21,054	40,435	12,091	42,656	47,809	40	7,198	13*	390
<i>BCOR</i>	L179F	0*	0*	10*	2*	2*	0*	6*	0*	0*
<i>BICD1</i>	I636L	0*	146	0*	26*	14*	2*	0*	0*	0*
<i>CDH5</i>	R44C	9*	15,247	325	19*	11*	4*	12*	3*	6*
<i>CYP4F2</i>	D446E	19,579	178,774	13,066	200,808	214,570	2,127	53,894	139,321	1,287
<i>DNAH1</i>	F1386L	7,604	259	359	5,081	24,514	292	356	4,384	79,960
<i>ELL3</i>	L87H	3,915	466	149,999	22,580	65,852	71,106	104,968	205	2,610
<i>EMILIN2</i>	D717N	33,857	84,709	25,017	121,654	149,458	2,591	148,951	1,400	929
<i>FXR2</i>	G604E	64	592	32,939	17,982	14,441	605	98	16*	735
<i>GUCY2F</i>	D822Y	0*	262	25*	0*	0*	0*	0*	0*	0*
<i>HOXA5</i>	R218C	0*	0*	3*	17*	16*	0*	0*	0*	0*
<i>HPX</i>	C366S	0*	2,152	9*	0*	54	0*	2*	2*	0*
<i>MAP1B</i>	D853Y	2*	2,067	664	4*	8*	1*	209	0*	0*
<i>MUC4</i>	A114V	35,652	17,281	76,231	104,247	35,781	4,018	16,755	700	44,224
<i>MYO1A</i>	G550R	8*	6*	9,956	6,923	482	6*	232	2*	18*
<i>OR2AP1</i>	L283F	0*	1*	2,889	1*	3*	159	2,070	0*	33
<i>PANX3</i>	V41I	86,040	67,808	126,143	238,941	11,570	32,028	84,713	43,114	74,922
<i>PAX2</i>	V319M	9*	71	1,188	13,973	7,770	17*	1,056	5*	9*
<i>PIRT</i>	V61E	307,344	167,407	758	5,056	11,826	258	687	164	508
<i>PRSS3</i>	S102Y	55,070	2,267	145,662	75,575	38,968	292,867	210,314	1,156	318,429
<i>TEX10</i>	R838L	113,253	177,776	139,014	15,248	8,896	157,617	246,950	507	93,955
<i>TRANK1</i>	E2772K	33,277	29,315	55,962	41,480	44,697	2,656	39,040	440	5,390
<i>ZNF599</i>	C181S	20,218	152	277	275	41,109	391	273	76	49,253
<i>ZNF777</i>	S10L	10*	3,096	2,705	7,832	5,456	11*	9,449	10*	224

Gene Target	Mutation	PIN-A Reads	PIN-B Reads	PIN-C Reads	PIN-D Reads	PIN-E Reads	PIN-F Reads	Normal Reads	Gp3 Reads	Gp4 Reads
Gp3-specific:										
<i>DNAH9</i>	D1547N	25,635	11,500	31,031	7,874	11,250	2,220	21,503	29*	4,296
<i>MSH6</i>	G971E	4,513	1,034	386	4*	3*	2*	254	0*	0*
<i>MUC16</i>	G13582S	301,734	293,295	414,034	631,091	442,378	260,708	398,295	299,998	239,397
Gp4-specific:										
<i>ABCA4</i>	A222V	30,637	41,550	28,978	178,514	129,252	3,297	40,363	61,308	582
<i>CDC42</i>	L104M	0*	6*	0*	0*	0*	0*	0*	0*	0*
<i>KDM4D</i>	R263C	35,819	68,151	44,731	5,473	17,437	17,377	40,658	83	1,709
<i>TEX15</i>	L1590F	27,494	1,003	16*	61	224	8*	20*	7*	19*
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Sequenced targets		20	27	25	22	23	18	24	14	20
Unsequenced targets		12	5	7	10	9	14	8	18	12

* Cases where read depth was below 33 reads and a false negative result cannot definitively be ruled out.