FAST TRACK

High Incidence of Somatic BAP1 Alterations in Sporadic Malignant Mesothelioma

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Background: Breast cancer 1-associated protein 1 (BAP1) is a nuclear deubiquitinase that regulates gene expression, transcription, DNA repair, and more. Several findings underscore the apparent *driver* role of *BAP1* in malignant mesothelioma (MM). However, the reported frequency of somatic *BAP1* mutations in MM varies considerably, a discrepancy that appeared related to either methodological or ethnical differences across various studies.

Methods: To address this discrepancy, we carried out comprehensive genomic and immunohistochemical (IHC) analyses to detect somatic *BAP1* gene alterations in 22 frozen MM biopsies from U.S. MM patients.

Results: By combining Sanger sequencing, multiplex ligation-dependent probe amplification, copy number analysis, and cDNA sequencing, we found alteration of *BAP1* in 14 of 22 biopsies (63.6%). No changes in methylation were observed. IHC revealed normal nuclear BAP1 staining in the eight MM containing wild-type *BAP1*, whereas no nuclear staining was detected in the 14 MM biopsies containing tumor cells with mutated *BAP1*. Thus, IHC results

were in agreement with those obtained by genomic analyses. We then extended IHC analysis to an independent cohort of 70 MM biopsies, of which there was insufficient material to perform molecular studies. IHC revealed loss of BAP1 nuclear staining in 47 of these 70 MM biopsies (67.1%).

Conclusions: Our findings conclusively establish *BAP1* as the most commonly mutated gene in MM, regardless of ethnic background or other clinical characteristics. Our data point to IHC as the most accessible and reliable technique to detect BAP1 status in MM biopsies.

Key Words: Sporadic malignant mesothelioma, Somatic *BAP1* mutation, multiplex ligation-dependent probe amplification, Malignant mesothelioma, BAP1.

(*J Thorac Oncol.* 2015;10: 565–576)

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Malignant mesothelioma (MM) is an aggressive tumor that arises from the mesothelial cells that form the lining of the pleural, pericardial, and peritoneal cavities.[1] In the United States, an estimated 3200 people are diagnosed with MM each year, with nearly 100,000 new cases expected to occur over the next 40 years.[2] The association between exposure to asbestos and to other mineral fibers and MM is well established.[3] Only a fraction of asbestos-exposed individuals develop MM, indicating that other factors may contribute to the development of the disease.[1] We have shown that in some families an extremely high susceptibility to develop MM is transmitted in an autosomal dominant fashion.[4] Recently, we identified germline mutations in the BRCA-associated protein 1 (*BAP1*) gene as the cause of a novel cancer syndrome characterized by a very high incidence of MM and uveal melanoma,[5,6] and benign atypical melanocytic lesions,[7] known as melanocytic BAP1-mutated atypical intradermal tumors (MBAITs),[8] and also by an elevated risk of other malignancies, such as cutaneous melanoma,[7] renal cell carcinoma,[9] cholangiocarcinoma,[10] basal cell carcinoma,[11] and possibly more.[5,6]

BAP1 is a nuclear deubiquitinase, which belongs to the ubiquitin C-terminal hydrolase family.[12] Among ubiquitin C-terminal hydrolase family members, BAP1 is unique because of its long C-terminal tail, which contains two nuclear localization signals (NLS1 at 656–661 and NLS2 at 717–722).[13,14] It has been postulated that BAP1 functions as a tumor suppressor,[6] possibly through three or more mechanisms, all requiring the nuclear localization of the BAP1

protein: (1) as component of the Polycomb repressive deubiquitinase complex, BAP1 deubiquitinates histone H2A, leading to transcriptional activation of genes that regulates cell growth[15]; (2) BAP1 acts as a transcription coregulator, associating with host cell factor-1, YY1, and E2F1 to induce transcription of genes involved in cell cycle regulation[12,16–18]; and (3) BAP1 contributes to the process of DNA repair.[6,19,20] Whether BAP1 also has some presently unknown cytoplasmic activity remains unknown.

Somatic *BAP1* mutations are found in sporadic MM (i.e., MMs that occur in individuals that do not carry germline *BAP1* mutations), as well as in other malignancies, although the frequency of *BAP1* mutations varies widely across different tumor types. Pena-Llopis et al.[21] detected somatic *BAP1* mutations in 14% of renal cell carcinoma specimens (24 of 176 tumors) using Sanger and whole-genome sequencing, whereas Harbour et al.[22] detected mutations in 84% of metastasizing uveal melanoma biopsies (26 of 31) using next-generation sequencing (NGS). In sporadic MM, by using Sanger sequencing, we found somatic *BAP1* mutations in 22% of U.S. Caucasian MM biopsies (4 of 18).[5] These results are in accordance with the results of Bott et al.[23] and Zauderer et al.,[24] who found 23% (12 of 53) and 20% (24 of 121) *BAP1* mutations in sporadic U.S. MM, also by Sanger sequencing. We described loss of BAP1 nuclear staining in MM tumor biopsies containing mutated *BAP1*.[5] We and Bott et al.[23] also noted loss of BAP1 nuclear staining in some MM biopsies that apparently had wild-type *BAP1* according to Sanger sequencing. Arzt el al.[25] revealed absence of BAP1 nuclear staining in 60% of 123 MM biopsies in a study that was based exclusively on immunohistochemistry (IHC). Yoshikawa et al.[26] found that *BAP1* was mutated in 61% of cell cultures (14 of 23) derived from pleural fluids of Japanese MM patients. In this study, Yoshikawa et al.[26] performed microarray-based comparative genomic hybridization (array CGH), Sanger sequencing, and real-time polymerase chain reaction (PCR) on DNA extracted from tumor cells established in tissue culture, after several subcultures. Instead, our study[5] and the studies by Bott et al.[23] and Zauderer et al.[24] were based on Sanger sequencing of tumor DNA from primary frozen tissue biopsies. The significant difference in frequency of *BAP1* mutations reported may due to differences in ethnicities of the patients studied, lack of sensitivity and/or reproducibility of Sanger sequencing or IHC in detecting *BAP1* mutations, procedure of tumor cell isolation and accumulation of novel *BAP1* mutations by MM cells in tissue culture,[26] lack of specificity of IHC, or some other differences in methodology. To investigate the basis of the discrepancies between these studies and to try to conclusively address this issue, we performed multiplex molecular analyses to comprehensively identify all possible genetic alterations of the *BAP1* gene in sporadic MM biopsies. We found that *BAP1* is mutated in more than 60% of MM specimens.

MATERIALS AND METHODS

Specimen Collection

Twenty-two MM frozen biopsies and matching normal leukocytes were obtained from the New York University (NYU) Langone Medical Center (HIP). MM samples were harvested during surgery and immediately frozen in liquid nitrogen.

An independent cohort of 70 formalin-fixed paraffin-embedded tissue slides was obtained from the National Mesothelioma Virtual Bank (NMVB) at the University of Pittsburgh. These latter specimens were used exclusively to extend BAP1 IHC analyses, as the amount of tissue available was insufficient to perform molecular studies. Specimens were de-identified before analysis. Sex, ethnicity, asbestos exposure, age at diagnosis, histology, and stage data were collected and are reported in the Supplementary Table 1A (Supplemental Digital Content 1, http://links.lww.com/JTO/A775).

Germline *BAP1* wild-type (WT) DNAs were obtained from healthy volunteers. Written and informed consent was obtained from all patients included in these studies according to the guidelines set forth by the institutional review boards.

Laser Capture Microdissection

Laser capture microdissection was performed to enrich tumor cells from frozen tissue specimens for DNA and RNA isolation. The detailed procedure is described in Supplementary material (Supplemental Digital Content 2, http://links.lww.com/JTO/A776).

DNA and RNA Extraction

DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) or QiAamp DNA Micro Kit (Qiagen 56304) as per manufacturer's instructions. Total RNA was extracted using mirVana miRNA isolation kit (Ambion, Austin, TX) as per manufacturer's instructions. Sample quality was evaluated by nanodrop. The 260/280 ratio was between 1.66 and 2.07 for DNA samples and between 1.94 and 2.15 for RNA samples.

Real-Time PCR

Total RNA was converted to cDNA using ImProm-II Reverse Transcription System (Promega, Madison, WI), following the manufacturer's instructions. A double-strand cDNA library was synthesized using the LD PCR kit (SMART cDNA Library Construction Kit; Clontech Laboratory, Mountain View, CA) according to the manufacturer's instructions.

DNA and RNA Sequencing Analysis

Conventional Sanger sequencing was performed as described.[5] Genomic *BAP1* PCR product sizes ranged from 560 to 670 base pairs (bp) with 100 to 150 bp overlap between primer sets. A similar primer design was employed for cDNA sequencing. Sequencing was conducted using the ABI 3730XL DNA Sequencer, at the Advanced Studies in Genomics, Proteomics and Bioinformatics facility at the University of Hawai'i at Manoa.

Multiplex Ligation-Dependent Probe Amplification Assay

Multiplex ligation-dependent probe amplification (MLPA) is a reliable technique extensively validated for

detection of medium and large gene alterations, which cannot be detected by conventional Sanger sequencing.[27,28] MLPA assay was performed according to the manufacturer's protocol (SALSA MLPA probemix P417-B1 BAP1; MRC-Holland, Amsterdam, The Netherlands). The detailed procedure is described in Supplementary material (Supplemental Digital Content 2, http://links.lww.com/JTO/A776) and Supplementary Figure 3 (Supplemental Digital Content 3, http://links.lww.com/JTO/A777).

Immunohistochemistry

IHC analysis of BAP1 protein expression was performed as described using a mouse monoclonal anti-BAP1 antibody (C-4: Santa Cruz Biotechnology, Dallas, TX).[5] This antibody recognizes the epitope between aa 430 and 739; therefore, it is predicted to detect BAP1 wild-type and mutant forms that retain the NLS. We had previously reported that BAP1 is expressed in normal pleural cells.[8] We extensively validated this antibody for the detection of nuclear BAP1 on a number of normal human pleural samples—all showing nuclear staining in 100% of pleural cells, as well as on MM-derived cell lines in which *BAP1* gene status has been comprehensively characterized in our laboratory. Histologically normal pleura and liver biopsies from autopsies were used as positive and negative controls, respectively, for BAP1 nuclear staining. Liver biopsies were chosen because they were shown to contain negligible amounts of BAP1 (http://www.proteinatlas.org/ENSG00000163930-BAP1/tissue/liver). We confirmed that BAP1 does not stain the nuclei of liver cells.

Two U.S. board-certified pathologists with extensive experience in MM diagnosis (MC and AP) independently reviewed all IHC results and concurred with their interpretation. The patients' characteristics of the control materials are not known, and this information cannot be obtained due to Health Insurance Portability and Accountability Act (HIPAA) constraints.

Promoter Methylation Analysis

Tumor DNA was modified with sodium bisulfide by using EZ DNA methylation kit (ZYMO research, Orange, CA) according to the manufacturer's protocol. The detailed procedure is described in Supplementary material (Supplemental Digital Content 2, http://links.lww.com/JTO/A776).

Whole Gene Methylation Analysis

BAP1 gene body methylation analysis was performed using the Methyl Primer Express Software v1.0 (Applied Biosystems, Grand island, NY), as described in Supplementary material (Supplemental Digital Content 2, http://links.lww.com/JTO/A776).

TaqMan Copy Number Assay

TaqMan copy number assay was performed at the Genomics Shared Resource, University of Hawai'i Cancer Center, according to the manufacturer's instructions (Supplementary material, Supplemental Digital Content 2, http://links.lww.com/JTO/A776).

Statistical Analysis

We studied the relationship between *BAP1* mutation status and age, sex, ethnicity, asbestos exposure, and histology using Fisher's exact test. Epithelioid MMs were compared with all other histological types; MM from patients older than 65 years were compared with all the others; patients exposed to asbestos were compared with all the others. Overall survival was estimated using the Kaplan-Meier method. Patients alive at the end of the study were censored at the time of the last available follow-up. Using the log-rank test, we compared survival between MM patients with altered *BAP1* versus patients with wild-type *BAP1*.

RESULTS

Sanger Sequencing and IHC for BAP1

Sanger sequencing was performed on DNA extracted from laser microdissected tumor cells and from matching germline DNA isolated from blood leukocytes of each of 22 MM patients. Matching germline DNA was found to contain the wild-type *BAP1* gene in 22 of 22 samples. Sequencing analysis detected *BAP1* alterations in a total of 6 of the 22 MM biopsies tested (27.3%, Table 1), which is in accordance with our previous findings.[5] The 22 MM biopsies included 15 samples that had been tested previously by Sanger sequencing, whose DNA was re-extracted from a separate vial containing frozen tumor tissue, and seven samples not previously analyzed yet. All 22 samples were analyzed blindly to test for reproducibility. The results obtained using Sanger sequencing were very reproducible as the same four samples that had tested positive for *BAP1* mutations, NYU647, NYU658, NYU866, and NYU937 previously,[5] retested positive and the 11 samples that had tested negative[5] retested negative (Table 1). These findings indicated that Sanger sequencing of DNA from tumor cells laser microdissected from frozen MM biopsies is very sensitive and specific in detecting at least certain types of *BAP1* DNA alterations.

In addition, we identified two new *BAP1* mutations among the seven new MM biopsies, while five tested negative (*BAP1* wild type). A point mutation (G → T) was found at the end of intron 14 on the genomic DNA of NYU524 (Fig. 1*A*). cDNA sequencing of this region revealed a 70 nucleotide insertion of an extra sequence from intron 14 (Fig. 1*B*). A single G insertion in exon 14 was detected in MM NYU1024 by sequencing both genomic DNA and cDNA (Fig. 1*C*). This variant encodes a presumed truncated BAP1 protein (642aa) missing the NLS as a result of the frameshift mutation (Fig. 1*D*). Overall, Sanger sequencing detected *BAP1* mutations in 6 of 22 samples (27.3%) all predicted to result in truncated proteins lacking the NLS.

IHC showed complete lack of BAP1 nuclear staining in 100% of tumor cells of these six mutated samples. Unexpectedly, in eight additional biopsies that appeared to contain wild-type *BAP1* by Sanger sequencing, no nuclear staining was detected. Overall, 14 of 22 MM biopsies (63.6%) contained tumor cells lacking BAP1 nuclear staining. Instead, the nuclei of the tumor cells of the additional eight MM biopsies carrying wild-type

TABLE 1. Summary of Somatic BAP1 Alterations in Sporadic Malignant Mesotheliomas

Sample ID	2011 Nat Gen ID[a]	Genomic Sequencing	MLPA Analysis	DNA Copy Number Assay	cDNA Sequencing	BAP1 Nuclear Localization	Prediction of Amino Acid Change	Comprehensive Interpretation	Promoter Methylation
NYU-047	SP-024	WT	WNL	—	WT	(+)		Normal	1.7%
NYU-207		WT	Ex 7–13 deletion	Ex 7–13 deletion	Ex 7–13 deletion	(−)	p.Pro147fsX63[b]	Mutant	1.3%
NYU-217	SP-019	WT	WNL	—	WT	(+)		Normal	1.6%
NYU-269	SP-017	WT	WNL	—	WT	(+)		Normal	1.3%
NYU-517	SP-016	WT	WNL	—	WT	(+)		Normal	1.7%
NYU-524		Int 14 splicing site (52, 436, 887 G>T)	WNL	—	70 nucleotide insertion at Ex 14 and 15	(−)	p.Glu630fsX23	Mutant	1.5%
NYU-540	SP-025	WT	Entire gene deletion	Entire BAP1 deletion	—	(−)		Mutant	1.4%
NYU-559	SP-022	WT	Ex 4–17 deletion	Ex 4–17 deletion	—	(−)		Mutant	3.5%
NYU-647	SP-001	Ex 11 (52, 439, 219 C deletion)	WNL	—	Ex 11 C deletion	(−)	p.Ser341fsX21[b]	Mutant	1.8%
NYU-658	SP-018	Ex 17 (52, 436, 398–399 CG deletion)	WNL	—	Ex 17 CG deletion	(−)	p.Arg699fsX17	Mutant	1.4%
NYU-809	SP-011	WT	WNL	—	WT	(+)		Normal	1.5%
NYU-851	SP-012	WT	Ex 1, 4, 15 deletion	WT	Ex 11–17 deletion	(−)	p.Val335fsx27[c]	Mutant	1.3%
NYU-866	SP-013	Ex 16 (52, 436, 599–627 29-bp deletion)	Entire deletion of other allele	—	Insertion of Int 16	(−)	p.Ala683fsX3	Mutant	1.2%
NYU-929	SP-023	WT	WNL	—	WT	(+)		Normal	1.1%
NYU-937	SP-015	Ex 9 (52, 440, 352 G deletion)[d]; Ex 13 (52, 437, 664 C deletion)[d]	Ex 9 deletion; WNL	—	Ex 9 G deletion; Ex 13 C deletion	(−)	p.Val234fsX15[b]; p.Ile499fsX72	Mutant	1.7%
NYU-966		WT	Ex 1 and 4 deletion	WNL	Ex 12–15 deletion	(−)	Deletion between 375 and 633	Mutant	1.8%
NYU-1017	SP-009	WT	WNL	—	WT	(+)		Normal	1.5%
NYU-1024	SP-026	Ex 14 (52, 437, 281- 52, 437, 282 G insertion)	Ex 10, 12, 14, 16, and 17 deletion	—	Ex 14 G insertion	(−)	p.Pro588fsx55	Mutant	2.1%
NYU-1250		WT	WNL	—	Deletion in Ex 13	(−)	p.Tyr418fsx113	Mutant	1.5%
NYU-1306		WT	Triplication	Increase of Ex 2, 4, 7, 12, 15, and 17	Ex 12, 13, 14 deletion	(−)	p.Lys368fsx33[c]	Mutant	1.4%
NYU-1359		WT	WNL	—	WT	(+)		Normal	1.6%
NYU-1419		WT	Random exon deletion	Decrease of Ex 2, 4, 7, 15, and 17	WT	(−)		Mutant	1.4%

None of these point mutations and alterations were detected in germline DNA samples from these patients. Average promoter methylation % is shown. BAP1 protein nuclear expression are represented (+) positive and (−) negative. Triplication is gain of gene copy number (three copies).

[a]Sample ID from Testa et al.[5] (Supplementary Table 1A, Supplemental Digital Content 1, http://links.lww.com/JTO/A775).

[b]Putative BAP1 mutant form may not be detected by BAP1 antibody.

[c]Putative BAP1 mutant form may be detected by BAP1 antibody.

[d]Point mutations were detected in each allele.

MLPA, multiplex ligation-dependent probe amplification; WT, wild-type sequence; WNL, within normal limit (duploid, two copies); —, not tested.
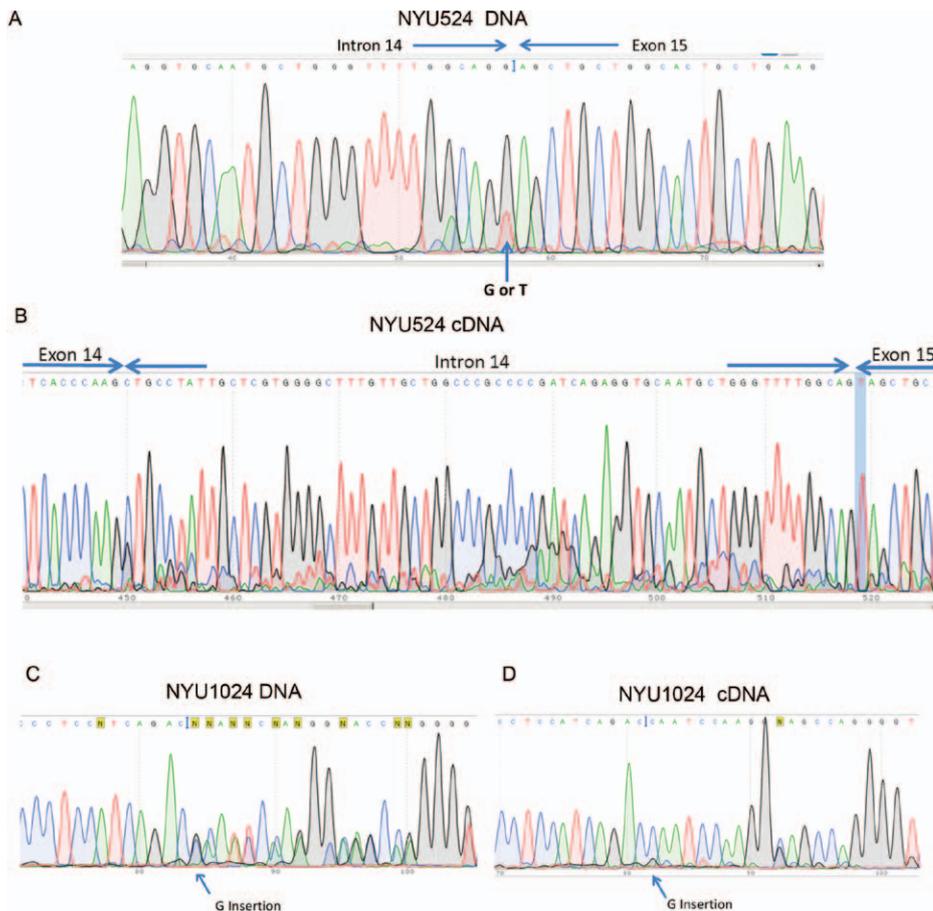
FIGURE 1. Point mutations of *BAP1* genes detected by Sanger sequencing. *A*, Tumor NYU524. Genomic DNA sequencing shows point mutation (G → T) at the splicing site at the end of intron 14. *B*, cDNA sequencing revealed a 70 nucleotide insertion from part of intron 14 (c.1891_1892Ins 70, p.Glu630fsX23). *C*, Tumor NYU1024. Genomic sequencing displayed G insertion in exon 14 (c.1762_1763 Ins G). *D*, cDNA sequence of the corresponding region (p.Pro588fsx55).

BAP1, as detected by Sanger sequencing, uniformly (all tumor cells) stained for BAP1 (Fig. 2).

MLPA, TaqMan Copy Number Analysis, and cDNA Sequencing Detected a Number of Previously Unidentified Gross Alterations of *BAP1* in Somatic MM

Tumor DNA from NYU540, NYU559, NYU1419, NYU207, NYU851, NYU966, NYU1306, and NYU1250 appeared to contain wild-type *BAP1* according to Sanger sequencing, while none of the tumor cells displayed nuclear BAP1 staining (Fig. 2). NYU540 tumor DNA displayed homozygous deletion of the entire *BAP1* gene by MLPA analysis (average normalized dosage quotient [NDQ] of all exon = 0.387; Fig. 3*A*). This deletion was confirmed by TaqMan copy number analysis (Fig. 3*B*). NYU559 tumor DNA showed a homozygous deletion from exon 4 to exon 17 by MLPA analysis (exons 4–17 average NDQ = 0.267; Fig. 3*C*, *D*). MLPA also revealed a homozygous deletion of the *BAP1* gene in NYU1419, with the lowest NDQ value of 0.27 for exon 12 (Fig. 3*E*). These results were confirmed by TaqMan copy number assay (Fig. 3*F*). A 3919-bp deletion spanning exon 7 to 13 was detected in NYU207 tumor DNA by MLPA analysis and TaqMan copy number assay (NDQ value of 0.407 at exon 12; Fig. 3*G*, *H*), which was further confirmed by genomic DNA sequencing using primers specifically designed to detect this deletion (Supplementary Figure, Supplemental Digital Content 4, http://links.lww.com/JTO/A778). cDNA sequencing (c.437_1729del 1293) revealed that this deletion would result in a putative truncated protein (210 aa) lacking the NLS.

Three additional MM biopsies (NYU851, NYU966, and NYU1306) appeared to contain wild-type *BAP1* by Sanger sequencing, yet they lacked nuclear BAP1 staining (Fig. 2). These three tumors displayed various types of copy number alterations in *BAP1* exons by MLPA. As shown in Figure 4, NYU851 tumor DNA showed low NDQ values in exons 1, 4, and 15 (Fig. 4*A*). Aberrant splicing was found between exon 8 and exon 9 and cDNA sequencing identified the deletion of 1820 nucleotides from 1003 to 2822 (c.1003_2822 del 1820; Fig. 4*B*). NYU966 showed decreased copy number of exon 1 and exon 4 (Fig. 4*C*), while cDNA sequencing identified a transcript with deletion of 769 nucleotides from 1128 to 1896 (c.1128_1896del 769; Fig. 4*D*). An increase in exon copy number was detected in multiple *BAP1* exons using MLPA in tumor NYU1306, particularly exons 1 to 3, 7 to 9, and 10 to 17 showing a NDQ value above 1.4 (Fig. 4*E*). This copy number increase was confirmed by TaqMan Assay (Supplementary Figure, Supplemental Digital Content 5, http://links.lww.com/JTO/A779). Moreover, NYU1306 cDNA sequencing identified a deletion of 791 nucleotides from 1101 to 1891 leading to a putative truncated *BAP1* (c.1101_1891 del 791, 400aa; Fig. 4*F*).
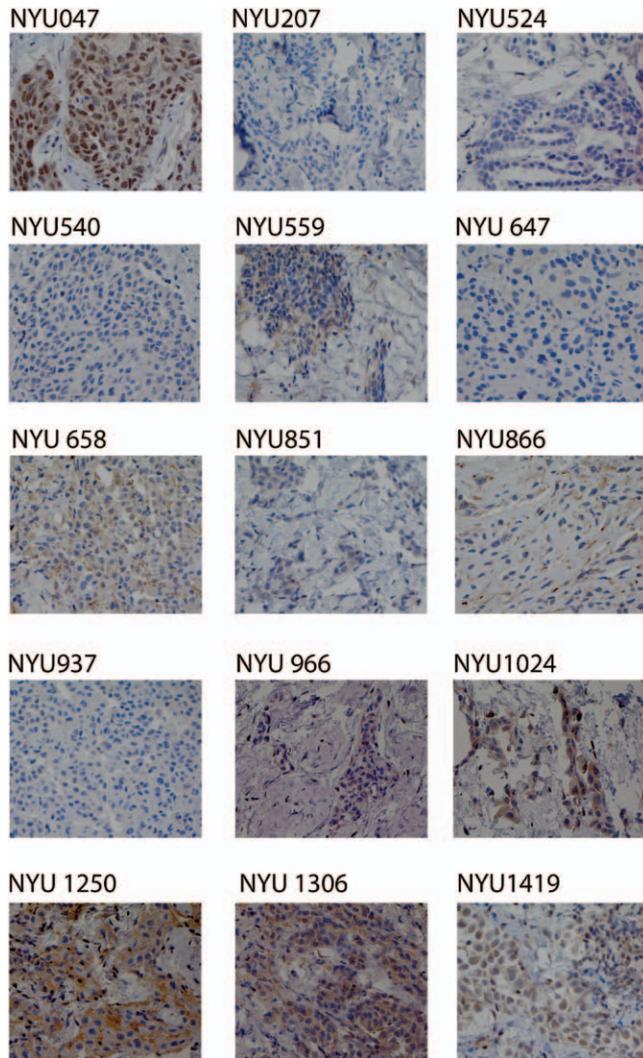
FIGURE 2. Detection of BAP1 protein expression in sporadic malignant mesothelioma tumor samples by immunohisto-chemistry. Wild-type *BAP1* tumor sample NYU047 shows strong nuclear staining in all tumor cells. All samples carrying alteration of *BAP1* gene are shown (photomicrographs magnification, 400×).

NYU1250 also lacked nuclear BAP1 staining (Fig. 2). However, in this sample, neither Sanger sequencing nor MLPA could identify *BAP1* alterations. cDNA sequencing detected a splicing mutant (c.1250_1371 del 122) in which the end of exon 12 was juxtaposed to the middle of exon 13 (Fig. 4*G*). A cryptic splicing site AG was found at the junction, which may generate a putative BAP1 protein of only 531 amino acids lacking the NLS (p.Tyr418fsx113; Fig. 4*H*). This splicing variant has not been reported previously (National Center for Biotechnology Information and GeneCards from the Weizmann Institute of Science).

Three samples showed *BAP1* alterations using both, Sanger sequencing and MLPA. NYU937 showed a reduced NDQ of exon 9. Sanger sequencing revealed a point mutation in the region of the MLPA probe for exon 9 (Supplementary Figure, Supplemental Digital Content 3, http://links.lww.

com/JTO/A777). MLPA also detected allele deletions in NYU tumor biopsies 866 and 1024 (Supplementary Figure, Supplemental Digital Content 6, http://links.lww.com/JTO/A780), which were shown by Sanger sequencing to contain DNA mutations in the other allele and lacked BAP1 nuclear staining (Figs. 1*C*, 2). Together these analyses revealed loss of heterozygosity (LOH) for these two samples.

No deletions were detected by MLPA, or by other techniques, in MM samples NYU047, NYU0217, NYU269, NYU517, NYU809, NYU929, NYU1017, and NYU1359. IHC revealed positive BAP1 nuclear staining in 100% of the tumor cells in these eight biopsies (Fig. 2 and Table 1).

BAP1 Promoter and Body Methylation Are Not Altered in MM

We sought to determine whether DNA methylation could be an additional mechanism leading to *BAP1* inactivation in sporadic MM. Transcriptional silencing may occur through methylation around the transcriptional start site; therefore, seven CpG loci, located approximately 450-bp upstream of ATG start codon, were used to check the methylation status of *BAP1* (Supplementary Figure, Supplemental Digital Content 7, http://links.lww.com/JTO/A781). Bisulfite-converted pyrosequencing revealed 86% to 98% methylation in a human high methylation genomic DNA control (Supplementary Figure, Supplemental Digital Content 7, http://links.lww.com/JTO/A781), whereas 2% to 4% methylation was detected in the low methylation control (Supplementary Figure, Supplemental Digital Content 7, http://links.lww.com/JTO/A781). We analyzed all 22 sporadic MM biopsies and found a consistently low level (1–4%) of methylation in all the samples. Methylation analysis of patient samples NYU047 and N524 is shown as an example (Fig. 5 and Table 1). These results indicate that hypermethylation is not a significant mechanism for *BAP1* inactivation in MM. Recently, gene body methylation was found positively correlated with gene expression.[29] Therefore, we investigated whether *BAP1* body methylation may influence *BAP1* gene expression. Using The Cancer Genome Atlas (TCGA) database, we analyzed *BAP1* methylation in several tumor types, at 19 sites spanning the entire *BAP1* promoter, gene, and 3′ untranslated region (Fig. 5*C*). Four probes located in the body region and 3′ untranslated region were uniformly heavily methylated in all nonmalignant and malignant tissues examined. The remaining 15 probes including all the ones located in the promoter/exon 1 region were completely or nearly completely unmethylated in all tissue samples examined (Fig. 5*C* and Supplementary Tables, Supplemental Digital Content 8, http://links.lww.com/JTO/A782, and Supplemental Digital Content 9, http://links.lww.com/JTO/A783), support the interpretation that methylation plays no role in the inactivation of the *BAP1* gene.

Extended IHC Analyses on a Separate MM Cohort

Our genetic results were very consistent with BAP1 IHC results, as all *BAP1* gene alterations detected resulted in loss of BAP1 nuclear staining by IHC. Thus, IHC appeared
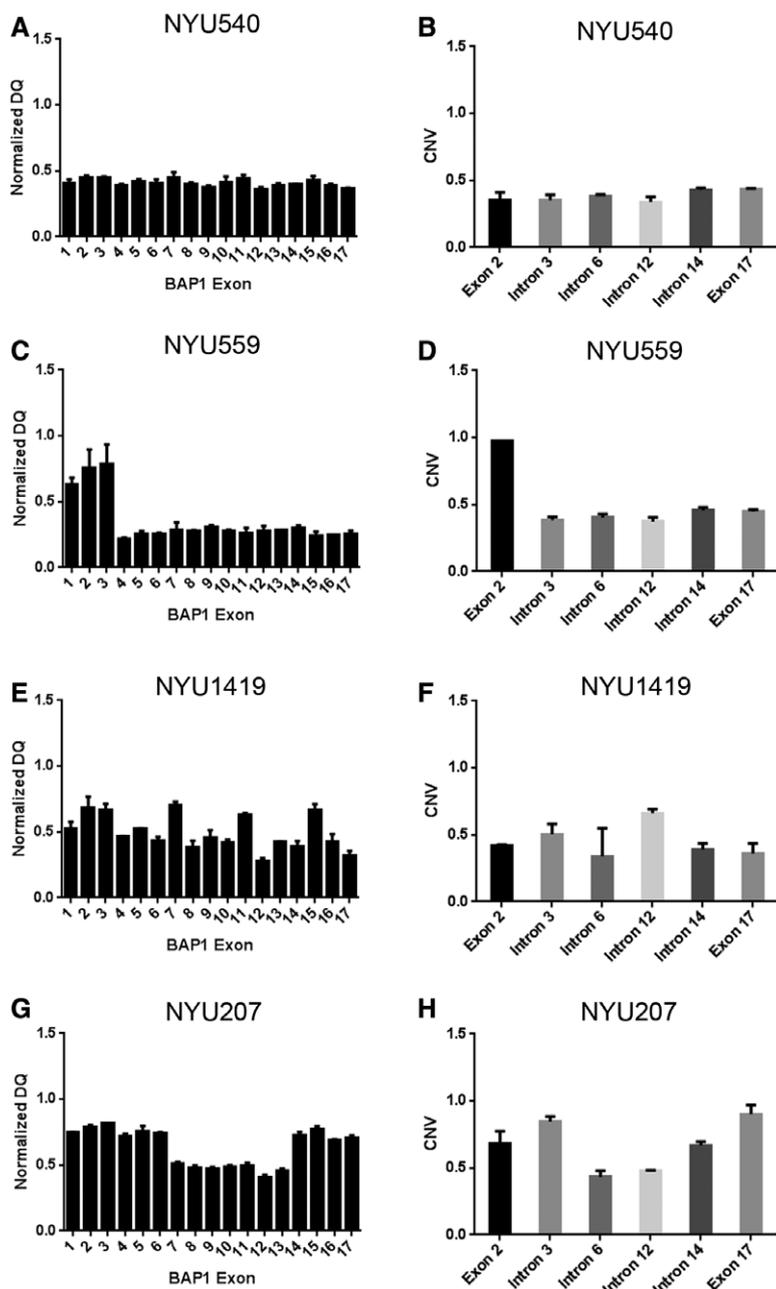
FIGURE 3. Gross *BAP1* gene alterations detected by MLPA and TaqMan copy number assays. MLPA assay is measured at 17 points, one each on 17 exons of *BAP1* gene. TaqMan copy number assay is measured at six points on *BAP1* gene; one each on exons 2, 3, 6, 12, 14, and 17. NYU540 MLPA and TaqMan data (*A, B*), NYU559 MLPA and TaqMan data (*C, D*), NYU1419 MLPA and TaqMan data (*E, F*), and NYU207 MLPA and TaqMan data (*G, H*). MLPA = multiplex ligation-dependent probe amplification.

a reliable method to identify MM biopsies containing mutated *BAP1*.

To extend these results, we performed BAP1 IHC on unstained paraffin embedded glass slides from an independent, annotated cohort from the NMVB (Supplementary Table, Supplemental Digital Content 1, http://links.lww.com/JTO/A775) consisting of a total of 70 cases of U.S. MM biopsies, of which we did not have additional material to conduct genetic analyses. This cohort included 63 Caucasian and seven non-Caucasian cases. We found loss of BAP1 nuclear staining in 47 of all 70 MM (67.1%) and in 44 of the 63 Caucasian MM biopsies (69.8%) (Supplementary Table, Supplemental Digital Content 1, http://links.lww.com/JTO/A775).

Patients' Demographics and Possible Clinical Correlations

Overall, the MM patients studied appeared to have received similar treatments as 45% of BAP1-altered and 44% of patients with normal BAP1 had surgery, and 35% of BAP1-altered and 31% of patients with normal BAP1 received chemotherapy (Supplementary Table, Supplemental Digital Content 1, http://links.lww.com/JTO/A775); thus, it is unlikely that treatment influenced some of the results. We did not find significant relationships between frequency of BAP1 alterations and sex, age of diagnosis, ethnicity, or history of asbestos exposure (Supplementary Table, Supplemental Digital Content 1, http://links.lww.com/JTO/A775). Patients
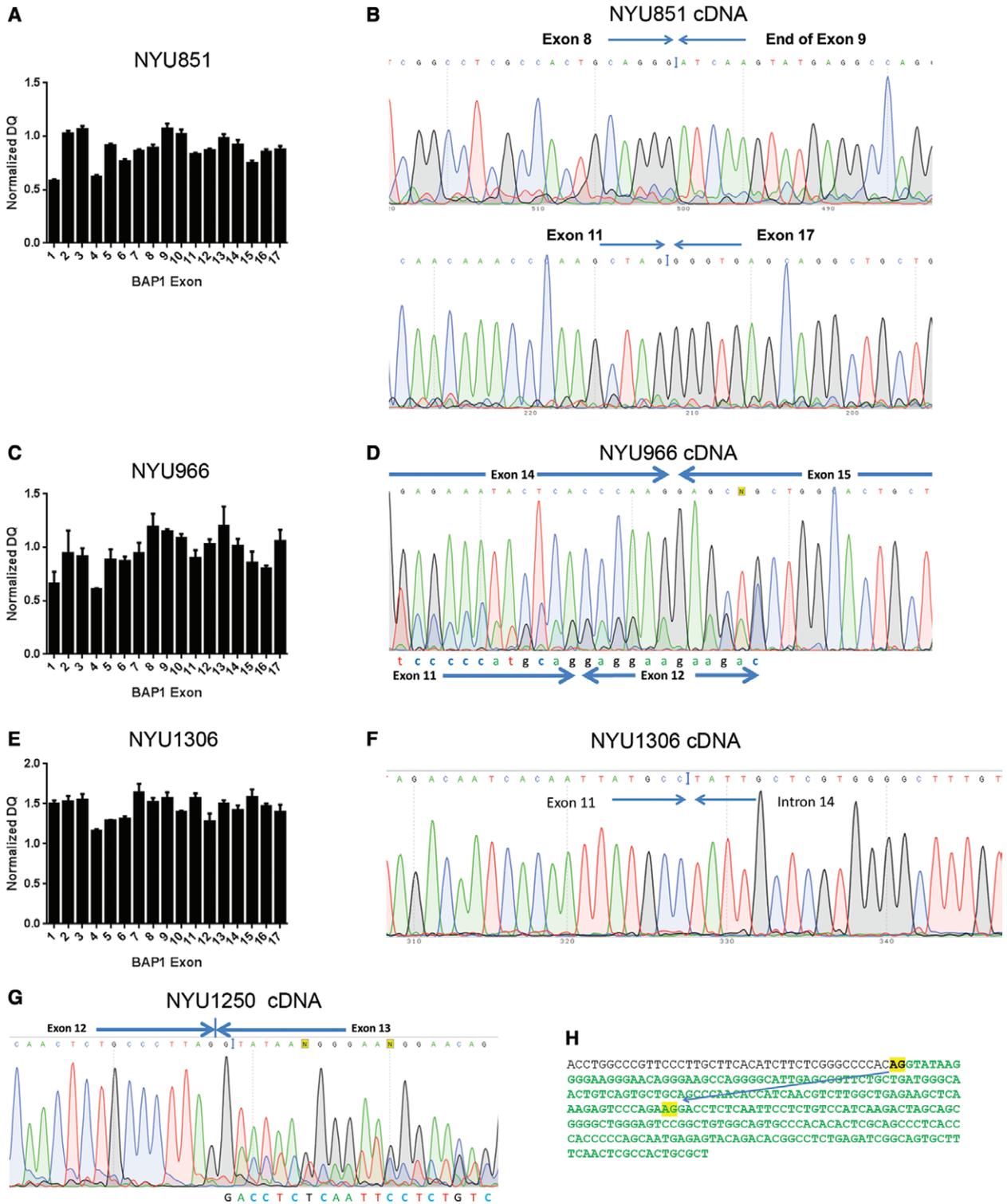
FIGURE 4. Detection of aberrant forms of *BAP1* by MLPA and cDNA sequencing. *A*, Tumor NYU851. MLPA assay detected deletions of exons 1, 4, and 15. *B*, NYU851 cDNA sequencing revealed two aberrant splicing at exons 8–9 and exons 11–17 in one allele. *C*, MLPA on NYU966 showed deletion of exons 1 and 4. *D*, Tumor NYU966 cDNA sequencing revealed a cDNA with skipping of most of exons 12, 13, 14, and 6 bp of exon 15 as well as a wild-type cDNA (exons 14 and 15 shown). *E*, MLPA on NYU1306 detected triplication, copy number increase to three copies, in most exons. *F*, NYU1306 cDNA sequencing revealed a deletion of exons 12 and 13. *G*, NYU1250 cDNA sequencing revealed a new splicing isoform of *BAP1* due to a 121-bp deletion in exon 13. *H*, Aberrant usage of cryptic splicing site AG, found in NYU1250, is shown by arrow on reference DNA sequence at intron 12 and exon 13. Nucleotides in green indicate the exon sequence. MLPA = multiplex ligation-dependent probe amplification.
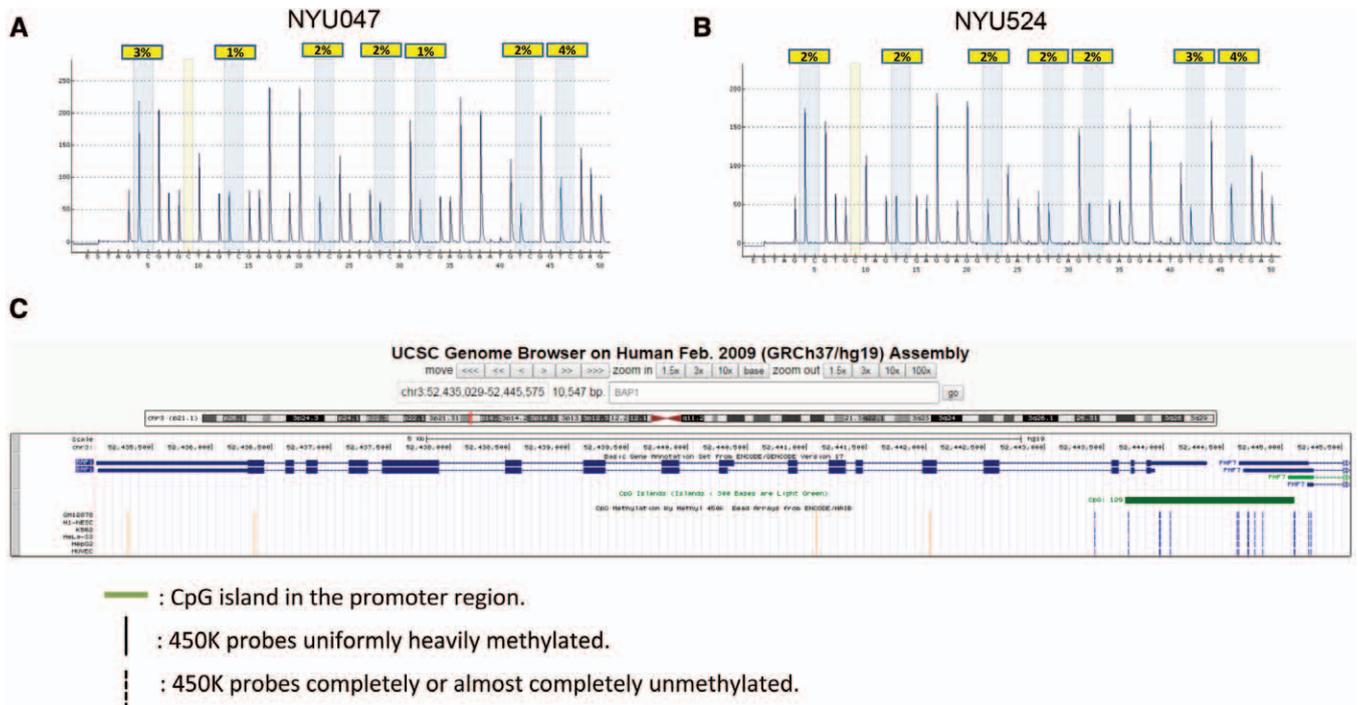
FIGURE 5. *BAP1* promoter and body methylation analysis. *A*, Sample NYU047. *B*, Sample NYU524. Low *BAP1* promoter methylation was detected by pyrosequencing in all sporadic MM samples tested. Pyrosequencing values detected in MM tumor samples are as low as the normal control DNA. Two representative samples are shown, where yellow highlights indicate the percentage of methylation at each site. *C*, The Cancer Genome Atlas portal database (https://tcga-data.nci.nih.gov/tcga/) was examined for methylation at *BAP1* gene, which was covered by 19 probes (data generated by use of the Infinium HumanMethylation450 BeadChip). MM = malignant mesothelioma.

with altered BAP1 had similar survivals than those with normal BAP1 (Supplementary Figure, Supplemental Digital Content 10, http://links.lww.com/JTO/A784). This was expected as each cancer contains many genetic alterations and it would be highly unlikely that mutations of a single gene influence prognosis. There was an apparent increased frequency of BAP1 alterations in patients with epithelial type MM versus other histological subtypes. However, the possible significance of all of these clinical correlates is tempered by the small sample size.

DISCUSSION

Using an integrated molecular approach that included Sanger sequencing, MLPA, TaqMan copy number analysis, messenger RNA sequencing, and IHC (Supplementary Figure, Supplemental Digital Content 11, http://links.lww.com/JTO/A785), we conclusively demonstrate that the majority (14 of 22, 63.6%) of sporadic MMs contain somatic *BAP1* mutations. Our study underscores the need of using distinct technical approaches to identify the nature of the different types of *BAP1* mutations possibly causing lack of BAP1 nuclear staining.

Our results indicate that the use of a single molecular technique is insufficient to detect all types of *BAP1* alterations. Although Sanger sequencing is reliable to detect single point mutations, which are various and frequent in MM and other cancer types,[5,8,21,26,30,31] the MLPA assay is much more reliable

to detect large deletions.[32] We found that Sanger sequencing was very reliable and reproducible in detecting *BAP1* point mutations and small deletions in preparations of DNA from laser dissected MM biopsies containing over 80% purity of tumor cells. However, this technique could not detect large DNA deletions. Conversely, MLPA was reliable to detect large exon gains and losses, even under conditions of relatively low sample purity (~50% of tumor cells). In this setting, MLPA is cost-efficient compared with array CGH, fluorescence in situ hybridization, and gene copy number assay.[33] However, MLPA could not detect single-nucleotide changes unless they fall within the MLPA probe target regions. In our study, 6 of 14 mutations were detected using conventional Sanger sequencing. Of these six mutations, five were not detected by MLPA because the mutated regions were small and not covered by the MLPA probe. On the other hand, seven additional aberrant forms of the *BAP1* gene were identified by the MLPA assay. Since the regions of exon copy number changes in these seven biopsies are large, Sanger sequencing could not detect these mutations. For accurate detection of deletions of large exons, such as exon 13 of *BAP1*, several MLPA probes had to be used. Abnormal splicing forms were detected in one MM biopsy—in which both Sanger sequencing and MLPA had not found any alteration—by RNA sequencing. Introns contain splicing enhancers and silencers,[34–36] which may partially explain the presence of some aberrant splicing variants in these MM samples.

Six of 14 MM biopsies had homozygous *BAP1* deletions as determined by both genomic analyses and IHC. For the remaining eight additional mutated samples, lack of nuclear staining supports LOH. However, molecular studies could not definitely establish LOH, possibly because traces of contaminant normal cell DNA were present in the samples.

There was 100% concurrence between detection of some molecular alterations in 14 of 22 biopsies and absence of BAP1 nuclear staining (Table 1 and Fig. 2). Strong nuclear and weaker cytoplasmic BAP1 IHC reliably identified the eight MM biopsies containing wild-type BAP1 from 14 biopsies containing mutated BAP1, whose nuclei did not stain for BAP1. Among the 14 mutated biopsies, seven contained tumor cells lacking BAP1 staining entirely (Fig. 2). Cytoplasmic BAP1 tumor cell staining was detected in seven biopsies with genetically mutated *BAP1* (Fig. 2). Cytoplasmic staining can be expected when mutations result in a truncated BAP1 protein lacking the NLS or bearing mutations in the catalytic subunit that prevent BAP1 auto-deubiquitination which is required for nuclear localization.[37] All mutated forms of *BAP1* detected here and in previous publications fall in this category and, therefore, are expected to lead to similar biological effects, as they all cause complete BAP1 protein loss or BAP1 isoforms sequestered in the cytoplasm and thus loss of BAP1 nuclear activity.

These results suggest that IHC may be the most reliable and accessible method to identify MM biopsies that harbor *BAP1* genetic alterations, while much more expensive and time-consuming genetic approaches can be used to (1) confirm the IHC findings, (2) detect heterozygosity, and (3) understand the mechanisms leading to loss of BAP1 expression.

To this date, all BAP1 activities have been related to nuclear localization.[10,13,14] Analysis of a separate cohort of MM biopsies from the NMVB revealed lack of BAP1 nuclear staining in 47 of 70 biopsies (67.1%), confirming the high frequency of *BAP1* mutations in U.S. MMs. Of note, in this cohort, six specimens contained a mixed tumor cell population, some with and some without BAP1 nuclear staining, possibly a result related to intratumoral heterogeneity and/or polyclonality.[38,39]

Promoter methylation is a common mechanism of tumor suppressor gene inactivation in several MM and other tumor types.[40,41] For example, Ras association (RalGDS/AF-6) domain family member 1 (RASSF1) and E-cadherin promoter hypermethylation was detected in MM patients.[42,43] High expression of DNA methyltransferases (DNMT1) was detected in pleural MM.[44] However, we observed no differences in methylation of the *BAP1* promoter or gene body. Similarly, no *BAP1* methylation alterations were found in clear cell renal carcinoma.[45] Whole-body methylation was also examined, considering the increasing volume of publications documenting a role of body methylation in gene expression.[29] While methylation data on MM are not available yet within the TCGA database, the data collected from multiple other tumor types indicate that methylation plays no role in the inactivation of the *BAP1* gene. In summary, DNA methylation does not seem to be a mechanism that contributes to *BAP1* inactivation in sporadic MM.

Although the information of demographics, histology, and survival is presented, we are not elaborating about possible clinical correlation with BAP1 status as the relatively small size of this cohort may bias interpretation.

In summary, our results demonstrate that: (1) *BAP1* inactivation in MM is achieved primarily by DNA mutation and changes in exon copy number; (2) different types of mutations lead to BAP1 lacking nuclear localization as shown by absence of BAP1 nuclear staining in all of the mutated samples and; (3) ethnicity does not seem to influence the frequency of BAP1 alterations.

Previously cyclin-dependent kinase inhibitor 2A/2B (*CDKN2A/2B*) and neurofibromin 2 (*NF2*) have been considered the most commonly mutated tumor suppressor genes in MM as they are found mutated in 30% to 50% and 35% to 40% of human MM biopsies, respectively.[46,47] However, the high percentage of *BAP1* mutations that we found in sporadic MM (>60%) now identifies *BAP1* as the most commonly mutated gene in this malignancy.

Our findings are supported by two recent NGS studies of the MM genome by Guo et al.[48] and Lo Iacono et al.[49] These two NGS studies revealed that various inactivating mutations occur randomly and are rarely shared among MM biopsies, with the exception of *BAP1* that was found mutated in 41%[48] and 58%[49] of MMs, respectively, and to a lesser extent *NF2*, *CDKN2A*[48,49], and possibly *CUL1*.[48] Lo Iacono et al.[49] also performed IHC studies and found that 52% of 116 MM biopsies from 116 patients stained for nuclear BAP1, an indication of normal BAP1 activity, whereas 48% did not, an indication of mutated *BAP1*.[49] BAP1 staining correlated with presence/absence of DNA mutations ($p = 0.001$)—of note in the same study *NF2* staining did not—independently supporting our findings that IHC is a reliable technology to identify *BAP1* mutations.[49]

Guo et al.[48] performed whole-exome sequencing of 22 MM biopsies from 22 MM patients and found 490 mutated genes of which 447 were mutated only in one biopsy. *BAP1* alterations were the most frequent mutations as they were detected in 41% of the 22 biopsies they studied, followed by CDKN2A and NF2. Six of these 22 MM biopsies were also included in our study (NYU269, NYU517, NYU647, NYU658, NYU929, and NYU937), allowing a direct comparison of the sensitivity of the technology used to detect *BAP1* mutations. In accordance with our results, Guo et al.[48] found that samples NYU269, NYU517, and NYU929 contained wild-type *BAP1*. They also detected the identical *BAP1* mutation in sample NYU937 (p.Ile499fsX15). However, although we detected homozygous mutations in samples NYU647 and NYU658 by Sanger sequencing—further confirmed by lack of nuclear staining by IHC—Guo et al.[48] did not (Table 1). This discrepancy may be due to the limitations of the NGS approach, which can produce base substitution errors, and to the fact that differently from Guo et al.,[48] we laser microdissected tumor cells from the biopsy, thus decreasing/eliminating the background of contaminating normal cells that contain wild-type *BAP1*. Alternatively, this discrepancy may be due to intratumor heterogeneity[38] and/or polyclonality.[39]

Our results and those from these two recent NGS MM studies underscore the apparent *driver* role of *BAP1* in MMs

and point at BAP1 as a potentially useful therapeutic target. In addition, somatic mutations in *BAP1* are present in other malignancies[6]; thus, therapies to restore BAP1 activity are of potential relevance to many cancer patients.

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