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KRAS (but not BRAF) mutations in ovarian serous borderline tumor are associated with recurrent low-grade serous carcinoma

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Abstract

BRAF and *KRAS* mutations in ovarian serous borderline tumors (OSBTs) and ovarian low-grade serous carcinomas (LGSCs) have been previously described. However, whether those OSBTs would progress to LGSCs or those LGSCs were developed from OSBT precursors in previous studies is unknown. Therefore, we assessed *KRAS* and *BRAF* mutations in tumor samples from 23 recurrent LGSC patients with known initial diagnosis of OSBT. Paraffin blocks from both OSBT and LGSC samples were available for 5 patients, and either OSBT or LGSC were available for another 18 patients. Tumor cells from paraffin-embedded tissues were dissected out for mutation analysis by conventional polymerase chain reaction (PCR) and Sanger sequencing. Tumors that appeared to have wild-type *KRAS* by conventional PCR–Sanger sequencing were further analyzed by full COLD (coamplification at lower denaturation temperature)-PCR and deep sequencing. Full COLD-PCR was able to enrich the amplification of mutated alleles. Deep sequencing was performed with the Ion Torrent personal genome machine (PGM). By conventional PCR–Sanger sequencing, *BRAF* mutation was detected only in one patient and *KRAS* mutations were detected in 10 patients. Full COLD-PCR deep sequencing detected low-abundance *KRAS* mutations in eight additional patients. Three of the five patients with both OSBT and LGSC samples available had the same *KRAS* mutations detected in both OSBT and LGSC samples. The remaining two patients had only *KRAS* mutations detected in their LGSC samples. For patients with either OSBT or LGSC samples available, *KRAS* mutations were detected in 7 OSBT samples and 6 LGSC samples. To our surprise, patients with the *KRAS G12V* mutation appeared to have shorter survival times. In summary, *KRAS* mutations are very common in recurrent LGSC, while *BRAF* mutations

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are rare. The findings indicate that recurrent LGSC can arise from proliferation of OSBT tumor cells with *or* without detectable *KRAS* mutations.

Keywords

Ovarian low-grade serous carcinoma; Serous borderline tumor; *KRAS* mutation; *BRAF* mutation; *KRAS* G12V; Full COLD-PCR; Deep sequencing

Introduction

The progression of ovarian serous borderline tumor (OSBT) to ovarian low-grade serous carcinoma (LGSC) is supported by clinical, pathological, and molecular evidence, although LGSC might also develop *de novo* [1-9]. Most OSBTs are diagnosed at an early stage and can be surgically cured [10-12]. In a series of patients with advanced OSBT, recurrent or persistent disease after surgery was observed in 29-44% of the patients, and 10-25% of the patients subsequently died of the disease [13-15]. Prognosis was excellent if the tumor recurred as a SBT. However, in 26-70% of the patients (depending on the length of the follow-up period), the tumor recurred as an LGSC, and more than 70% of these patients eventually died of the disease [14,15].

The molecular progression of OSBT has been unclear [16]. It is believed that its pathogenesis begins with serous cystadenoma/adenofibroma, which develops progressively into atypical proliferative serous tumor (typical serous borderline tumor), non-invasive micropapillary serous carcinoma (micropapillary serous borderline tumor) and subsequently invasive low-grade serous carcinoma. While cystadenoma/adenofibroma is assumed to arise from epithelial inclusion glands in the ovary, the origins of epithelial inclusion glands is controversial. Besides from the ovarian surface epithelium, epithelial inclusion glands might also originate from the fallopian tube from recent studies [17]. Thus, early precursors in the LGSC pathway, besides OSBT and endosalpingiosis, may also arise directly from the fimbriae of fallopian tube [18].

Development of LGSC is associated with activation of the mitogen-activated protein kinase pathway, mutations in *BRAF* and *KRAS* and increase in DNA copy number aberrations [19-21]. *KRAS* and *BRAF* mutations have been found in both primary OSBT and primary LGSC [22-24]. More recent reports indicate that *BRAF* mutations are more common in OSBT and early-stage LGSC but rare in late stage LGSC [20,25-27]. However, whether those OSBTs would progress to LGSCs or those LGSCs developed from OSBT precursors in previous studies is unknown. Whole exome sequencing analyses of seven ovarian LGSC indicated that LGSC contains very few point mutations. The most frequently mutated genes were still *BRAF* and *KRAS* [28]. However, no analyses of *KRAS* and *BRAF* mutations in patient samples with recurrent LGSC from initial diagnosis of OSBT have been reported to date.

Sanger sequencing, which has been used in all previous studies of *BRAF* and *KRAS* mutations in OSBT and LGSC, has a limited ability to detect mutations that occur only in a small percentage of aggressive tumor cells [29]. To investigate whether small populations of

KRAS mutated cells occur in OSBT, we analyzed patient tissue samples by full COLD (coamplification at lower denaturation temperature)-PCR coupled with deep sequencing. We hypothesize that a subset of recurrent LGSC originates from OSBT having *KRAS* but not *BRAF* mutations. This may allow us to assess the risk of advanced stage OSBT that may progress as LGSC.

Materials and Methods

Tissue samples from patients

Paraffin blocks from 23 patients with a primary diagnosis of OSBT and subsequent recurrent LGSC were used. Both OSBT and recurrent LGSC tissue samples were available for 5 of the 23 patients; for the other 18 patients, either an OSBT tissue sample or a recurrent LGSC tissue sample (but not both) was available. In addition, 13 cases of advanced OSBT samples from patients who have no progressive disease were also obtained for comparison. This cohort of patients had a median follow-up of 155 months (interval: 78 to 372 months). All samples were retrieved from the archives of the Department of Pathology at The University of Texas MD Anderson Cancer Center. Specimens had been collected, archived, and managed under research protocols approved by the Institutional Review Board. Sections of 5- to 10-micron thickness were cut from formalin-fixed paraffin-embedded (FFPE) tissue samples. A hematoxylin-eosin-stained slide corresponding to each paraffin block was reviewed by two gynecologic pathologists (M.T.D. and A.M.) to confirm the diagnosis. For samples where the tumor was either large or localized in a specific area, the DNA was isolated by using the Pinpoint Slide DNA Isolation System (Zymo Research, Irvine, CA). For samples where the tumor was present as a thin layer or was dispersed in the tissue section, tumor cells were dissected out by using laser capture microdissection (AS LMD; Leica Microsystems, Buffalo Grove, IL). The DNA was subsequently isolated using the QIAamp DNA Micro kit (Qiagen, Valencia, CA).

Full COLD-PCR

Fifty n genomic DNA was amplified by using anograms of 1× high-fidelity buffer, 0.2 mM dNTPs (deoxyribonucleotide triphosphates), 0.3 μM primers, 0.1× LCGreen Plus dye (Idaho Technology, Salt Lake City, UT), 0.02 U/ml of Phusion high-fidelity DNA polymerase (Thermo Scientific, Waltham, MA). The primers were Kras187F (AGTCACATTTTCATTATTTTATTATAAGG) and Kras139R (AAACAAGATTTACCTCTATTGTTGGATCA). We first determined the melting temperature of this PCR fragment in samples containing either wild-type *KRAS* or *KRAS* with the *G12D* mutation by monitoring the dissociation in real time using the CFX96 real-time PCR instrument (Bio-Rad, Hercules, CA) under regular PCR conditions: 98°C for 30 s; 98°C for 10 s, 56°C for 20 s, 72°C for 10 s, fluorescence reading (35 cycles); ramp from 65°C to 98°C in 0.2°C increments for 5 s per cycle. Next, critical temperature was determined empirically by trying a stepwise gradient of temperatures below the melting temperature using the following full COLD-PCR conditions as described by Milbury et al. [30]: 98°C for 30 s; 98°C for 10 s, 56°C for 20 s, 72°C for 10 s, fluorescence reading (5 cycles); 98°C for 10 s, 70°C for 30 s, variable critical temperature for 10 s, 56°C for 20 s, 72°C for 10 s, fluorescence reading (35 cycles); ramp from 65°C to 98°C in 0.2°C

increments for 5 s per cycle. Dissociation curves from both regular PCR and full COLD-PCR yielded only a single species, an observation that indicated that the *KRAS* primers were not binding to the *KRAS* pseudogene. A critical temperature that still yielded enough PCR products for making a library for sequencing was then chosen. Final full COLD-PCR conditions for generating PCR products for sequencing were as follows: 98°C for 30 s; 98°C for 10 s, 56°C for 20 s, 72°C for 10 s, fluorescence reading (5 cycles); 98°C for 10 s, 70°C for 30 s, 79°C for 10 s, 56°C for 20 s, 72°C for 10 s, fluorescence reading (35 cycles), 72°C for 5 min. A similar procedure was used to determine the conditions for Braf full COLD-PCR. The Braf primers used were Braf Seq (GAAAATGAGATCTACTGTTTTCTTTA) and Braf 200R (GACAACTGTTCAAACCTGATGG). The final Braf full cold PCR conditions were as follow: 98°C for 30s; 98°C for 10s, 56°C for 20s, 72°C for 10s, fluorescence reading (5 cycles); 98°C for 10s, 70°C for 30s, 78°C for 10s, 56°C for 20s, 72°C for 10s fluorescence reading (35 cycles), 72°C for 5min.

Sanger and deep sequencing

Sanger sequencing of PCR amplicons was done at the Sequencing and Microarray Facility of MD Anderson Cancer Center. For deep sequencing, the PCR product was purified with Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN) and processed for sequencing with the Ion Xpress Plus Fragment library kit (Life Technologies, Carlsbad, CA). Briefly, the PCR product was end repaired, ligated to adaptors, nick repaired, and purified. The sample was then used as a template for emulsion PCR with the Ion Xpress Template 200 kit (Life Technologies). Emulsion PCR is a process by which DNA is clonally amplified onto beads (Ion Sphere particles) so that they can be sequenced by the Ion Torrent Personal Genome Machine (PGM) (Life Technologies). An enrichment step to select for Ion Sphere particles with DNA attached to them was performed to maximize the number of sequencing reads generated by the PGM. A sequencing primer and polymerase from the Ion Sequencing 200 kit (Life Technologies) were then added to the enriched, DNA-positive Ion Sphere particles, which were then placed into the Ion 314 chip (Life Technologies) for sequencing in the PGM. Mixed DNAs from cell lines RMUGS (*KRAS* wild type) and MCAS (homozygous *KRAS G12D* mutation) were initially used to evaluate the sensitivity of *KRAS* mutations detection by full COLD-PCR followed by deep sequencing with Ion Torrent PGM.

Deep sequencing data analysis

FASTQ format files generated from Ion Torrent were uploaded into CLC Genomics Workbench version 6.0 software (CLC bio, Cambridge, MA). Sequencing reads in FASTQ format were trimmed with a quality score limit of 0.01 and a maximum of 2 ambiguous nucleotides. Five bases were also trimmed from both the 3' end and the 5' end of each sequencing read. After trimming, sequencing reads were aligned to reference chromosome 12 (NC_000012) with a fraction length of 0.5 and a similarity score of 0.9. After mapping, variants were called on the basis of quality scores (Phred quality score >25) for mapped reads.

Survival analysis

Overall survival was calculated from the date of diagnosis to the date of death or last known follow-up. Survival times were estimated using the Kaplan-Meier method. The log-rank test was used to compare differences between survival curves. The Cox proportional hazards regression analysis was used to evaluate the relationship between *KRAS* mutations and overall survival after adjustment for differences in age. P-values less than 0.05 were considered statistically significant. Statistical analyses were performed using SPSS version 19.0 software (IBM, Armonk, NY).

KRAS variants and AZD6244 (MEK inhibitor) sensitivity in cancer cell lines

From the “Cancer Cell Line Encyclopedia (CCLE)” database by Novartis and Broad Institute [31], we extracted the *KRAS* mutation status and sensitivity to AZD6244 (selumetinib) data for 503 cancer cell lines.

Results

High sensitivity of full COLD-PCR and deep sequencing to detect KRAS and BRAF mutations

In full COLD-PCR, the mutated allele is preferentially amplified over the normal allele on the basis of an empirically determined critical temperature. No *KRAS* variant was detected in the negative-control RMUGS cell line DNA with 188,649 sequencing reads (see Supplementary material, Table S1) or DNA extracted from FFPE sample of normal tissue of patient 1 with 227,502 sequencing reads (see Supplementary material, Table S2). However, full COLD-PCR was able to preferentially amplify the *KRAS G12D* mutant allele approximately 3- to 5-fold relative to amplification by standard PCR. The number of sequencing reads with the *KRAS G12D* mutation increased from 1.34% (2500 of 187,177 reads) by standard PCR to 3.56% (2312 of 64,972 reads) by full COLD-PCR, as detected by deep sequencing analysis with Ion Torrent PGM using a DNA mixture with approximately 1% *KRAS G12D* mutated genomic DNA (1% MCAS DNA, 99% RMUGS DNA) (see Supplementary material, Table S1). Similarly, with a 0.1% initial input of *KRAS G12D* DNA, full COLD-PCR detected 469 reads (0.46%) with the *KRAS G12D* variant among 102,376 reads. For the initial analysis, each sample was run on a single Ion 314 chip, and >100,000 mapped reads (Phred quality score >20) were generated for each sample. Subsequently, two or three samples were bar-coded to run on the same Ion 314 chip, and >5000 mapped reads were generated for each sample.

BRAF and KRAS mutations

Supplementary Table S2 summarizes the results of the mutation analysis of *BRAF* codon 600 and *KRAS* codon 12 in DNA extracted from microdissected tumor cells. *BRAF* and *KRAS* mutations in paraffin block tissue samples from 23 patients with recurrent LGSC were first analyzed by standard PCR–Sanger sequencing. With Sanger sequencing, 10 (43%) of the 23 patients had a *KRAS* mutation in the OSBT sample (n=6, Patient 2, 6, 10, 16, 17, and 19), recurrent LGSC sample (n=5, Patient 1, 2, 4, 5, and 13), or both (n=1, Patient 2). One of the patients, Patient 9, had a *BRAF V600E* mutation in her OSBT sample. Of the five

patients with both an OSBT sample and a recurrent LGSC sample available for analysis, (Patient 1 through Patient 5; see Supplementary material, Table S2), three had detectable *KRAS* mutations only in the recurrent LGSC sample and one had a detectable *KRAS G12D* mutation in both samples, as detected by Sanger sequencing.

Since *KRAS* mutations were not detected by Sanger sequencing in the OSBT samples of three patients whose recurrent LGSC samples did have *KRAS* mutations by Sanger sequencing (Patient 1, Patient 4, and Patient 5; Supplementary Table S2 and Figure 1), we used full COLD-PCR and deep sequencing to determine whether the OSBT samples contained *KRAS* mutated cells in numbers too small to be detectable by Sanger sequencing. With full COLD-PCR, we were able to detect the mutated *KRAS* in the OSBT sample from Patient 1 but not in the OSBT sample from Patient 5 (Figure 1). Deep sequencing analysis of full COLD-PCR amplicons of DNA from Patient 1 and Patient 5 clearly demonstrated the presence of *KRAS* mutations at frequencies of 46% (*KRAS G12D*; 60252 of 130,510 reads) and 0.5% (*KRAS G12V*; 677 of 154,288 reads), respectively (see Supplementary material, Table S2). As a negative control, no *KRAS G12D* (0/227,502) was detected in FFPE DNA extracted from a matched normal tissue sample from Patient 1.

We then analyzed additional OSBT and LGSC samples that were shown by standard PCR Sanger sequencing to contain only wild-type *KRAS*. Using full COLD-PCR deep sequencing, we detected *KRAS* mutations for eight additional patients (patient 3, 8, 11, 12, 20, 21, 22, 23). Thus, 78% of the patients in our series (18 of 23) had tumor cells with *KRAS* mutations. For the five patients with both OSBT and LGSC samples, three had same *KRAS* mutations detected in both OSBT and LGSC samples and the remaining two patients had only *KRAS* mutations detected in the LGSC samples. For patients with either OSBT or LGSC samples available, mutations were detected in 7 OSBT samples and 6 LGSC samples. Besides the common *KRAS* mutations *G12D*, *G12V*, and *G12A*, we found the rare *KRAS* mutations *T20R*, *I21M*, *Y4N*, and *A18G*. Using the public server <http://mutationassessor.org> [32], we determined that mutations *T20R*, *Y4N*, and *A18G* have a functional impact similar to that of *G12D* or *G12V*. Other mutations were also detected, but at much lower frequencies (see Supplementary material, Table S3). As a control, 13 OSBT samples (stage 2 and 3) from patients with no progressive disease were also analyzed by full COLD-PCR for both *BRAF* and *KRAS* mutations. Eight samples had mutations (5 with *BRAF V600E*, 2 with *KRAS G12D* and one with *KRAS G12C*) while the remaining 5 samples had no detectable mutations (see Supplementary material, Table S4).

KRAS mutations and patient survival

KRAS mutations and clinical data for the 23 patients are listed in Supplementary Table S5. Patients were classified into three groups on the basis of their *KRAS* status: (1) *G12V* mutation, (2) *G12D* mutation, (3) wild type or rare variants (*T20R*, *I21M*, *Y4N*, and *A18G*). Overall survival time was calculated from the time of initial OSBT diagnosis to the date of death or last follow-up for recurrent LGSC. The median survival times of patients with *KRAS G12V* from initial OSBT diagnosis were significantly shorter than those of patients with *KRAS G12D* or those of patients with the wild type or rare *KRAS* variants ($P < 0.05$) (Figure 3). The median survival time of patients with *KRAS G12V* was 125 months, while

patients with *G12D* had a median survival time of 189 months. The median survival time of patients with wild-type *KRAS* or rare *KRAS* variants was 168 months. After adjustment for age in Cox regression analysis, patients with *KRAS G12V* mutation were still significantly associated with shorter survival time in comparison to the rest of the patients (HR: 4.77, 95% CI: 1.24-18.37, $P = 0.023$).

Cancer cell lines with *KRAS G12V* are more sensitive to AZD6244

Cancer cell lines from the Cancer Cell Line Encyclopedia database were categorized based on their *KRAS* variant status. These were further subdivided into sensitive or resistant to AZD6244 (selumetinib) (see Supplementary material, Table S6). The median IC₅₀ for these 503 cancer cell lines was 7.8 μM and the IC₅₀ at the fifteen percentile for these cancer cell lines was 0.66 μM . Approximately, 15% of patients were responders in an AZD6244 clinical trial [26]. Assuming that the response rate of these cancer cell lines was similar to that in clinical trial, we would expect 15% of these cell lines to be sensitive to AZD6244. Thus, a cell line with an IC₅₀ less than 0.66 μM is considered as sensitive. Thus, there were 76 AZD6244 sensitive cell lines and 427 resistant cell lines. Using Fisher Exact Test, we found that cell lines with *KRAS* mutation were more sensitive to AZD6244 than wild-type cell lines but has not reach statistical significance (Odd ratio = 1.59; $p = 0.148$; 95% confidence, 0.893-2.830). Ninety-three cell lines had *KRAS* mutations. The most common *KRAS* mutations were *G12D* (26 cell lines) and *G12V* (20 cell lines). The frequency of *KRAS G12D* and *KRAS G12V* in these cell lines is similar to clinical samples. Using data from the “Catalogue of Somatic Mutations In Cancer” database [33], of 26197 clinical samples with *KRAS* mutations, 9250 samples had *G12D* (35%) and 6190 samples had *G12V* (24%). Using Fisher Exact Test, cell lines with *KRAS G12V* were found to be more sensitive to AZD6244 than cell lines with wild-type *KRAS* with an Odd ratio of 4.13 ($p = 0.005$; 95% confidence interval, 1.617-10.541), while cell lines with *KRAS G12D* had an Odd ratio of only 1.13 ($p = 1$; 95% confidence interval, 0.374 – 2.813).

Discussion

The advent of targeted therapies has increased the importance of detecting minute numbers of malignant cells in tumors that have progressed to an advanced stage by acquiring additional driver mutations or clonal expansion. This study has demonstrated for the first time that COLD-PCR with deep sequencing can detect a small number of tumor cells (<1%) with *KRAS* mutations in FFPE tissue samples. Since tumor biopsies can be quite heterogeneous and regular PCR Sanger sequencing can reliably detect a mutation only if the mutation is present in at least 30% of the DNA molecules, low-abundance mutations will thus be missed. The use of the Sequenom MassARRAY system can further increase detection sensitivity to approximately 5% [34]. Here, we evaluated the use of COLD-PCR and sequencing methods for its analytic sensitivity and quantitative performance in FFPE solid tumor biopsy samples. With a high-fidelity polymerase, we have demonstrated that the original percentage of mutations can be preserved after PCR amplification. Using deep sequencing with Ion Torrent PGM, we were able to detect the presence of mutations in as few as 0.1% of the molecules in the original DNA sample.

BRAF and *KRAS* mutations in primary OSBT and LGSC have been previously reported [20,22-26]. However, our study is the first to examine patients with an initial diagnosis as advanced stage OSBT who recur as LGSC. A recent study by Grisham *et al* included 30 advanced stage OSBT and 19 LGSC [35]. However, whether these 19 LGSC were developed from OSBT is not clear, and whether the 30 advanced stage OSBT will progress as LGSC is also unknown. In our study, we have analyzed 36 patients with advanced stage OSBT (13 did not recur and 23 had progressed as LGSC). The median follow-up of patients with recurrent LGSC in this study was 125.5 months (range: 117-168 months). On the other hand, the follow-up time from Grisham's study was 35.9 months (range: 0.8 - 129.3), which might not be long enough to see a difference between the impact of *KRAS G12V* and *KRAS G12D* on survival. We implemented a highly sensitive COLD-PCR deep sequencing method to detect low-abundance *KRAS* mutations, for which conventional Sanger sequencing was not informative. Our results demonstrate that *KRAS* mutations are very common in recurrent LGSC (>70%). More importantly, we were able to detect *KRAS* mutated cells present in very small numbers in primary OSBT samples for the first time. This novel finding indicates that the development of LGSC was through the clonal expansion of minor *KRAS* mutated clone in OSBT. Although the number of samples in this study was limited, we have provided the first evidence that the *KRAS* mutations in patients with LGSC were probably present in these patients at the time of OSBT diagnosis.

Another interesting observation is that patients with the *KRAS G12V* mutation appeared to have shorter overall survival than did patients without this mutation. Although the patient number might be small, it is statistically significant. This finding is similar to *KRAS* mutation findings in the study of over 3000 colorectal cancer samples: of 12 different mutations in *KRAS* codons 12 and 13, only *KRAS G12V* was associated with poor overall survival [36]; Keohavong *et al* also found a strong trend toward a poor prognosis with *KRAS G12V* in lung adenocarcinomas [37]. However, the clinical outcome could be the result of treatment rather than natural disease progression. On the other hand, the frequency of *BRAF V600E* mutation is much higher in OSBT that did not recur, strengthening the argument that *BRAF* mutation may protect against progression to LGSC as we have discussed previously [20]. Genome-wide expression patterns of *KRAS* mutations in a colorectal cancer cell line and fibroblast cell indicate that *KRAS G12V* may confer a more aggressive phenotype by allowing cells to escape apoptosis and inducing angiogenesis via interleukin 8 [38,39]. Moreover, by querying the The Cancer Genome Atlas ovarian high-grade serous carcinomas study using the cBio Cancer Genomics Portal [40], we found that there were two cases of ovarian high-grade serous carcinomas with *KRAS* mutations (TCGA-24-2038 and TCGA-25-1316) – both have *KRAS G12V* and wild-type *TP53*. It is tempting to speculate that these two high-grade serous carcinomas with *KRAS G12V* might have evolved from low-grade serous carcinoma as observed previously [2,41,42]. These data support our observation that *KRAS G12V* may confer a more aggressive phenotype in serous borderline tumor and recurrent low-grade carcinomas.

In this study, we also detected many low-abundance *KRAS* variations; this could indicate that dominant clones (*G12D* or *G12V*) are selected during disease progression. Detection of activating *KRAS* mutations by full COLD-PCR deep sequencing will be a more sensitive and

reliable method than regular PCR Sanger sequencing for stratifying patients in clinical trials of drugs targeting the KRAS/RAF/MEK pathway because of tumor heterogeneity. The lack of a correlation between *BRAF* or *KRAS* mutations and response to selumetinib (AZD6244) in a recent LGSC clinical trial [26] could be a result of the use of bulk primary FFPE LGSC tissue and the lower sensitivity of the detection method. We analyzed eight patients who were treated with selumetinib at MD Anderson: two had *KRAS G12V*, one had *KRAS G12D*, one had *KRAS G12A*, and the other four had wild-type *KRAS*. Interestingly, the two patients with *KRAS G12V* mutation were both responders. This is in agreement with the selumetinib sensitivity data of 503 cancer cell lines in the “Cancer Cell Line Encyclopedia” study. Further investigation is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Sanger sequencing traces for OSBT samples from two patients and recurrent LGSC samples from the same patients. A. Standard PCR, LGSC. B. Standard PCR, OSBT. C. Full COLD-PCR, OSBT.

Figure 2. Hematoxylin-eosin-stained slides of OSBT samples and recurrent LGSC samples from two patients.

Figure 3.

Kaplan-Meier curves for three groups of patients with recurrent LGSC and different *KRAS* mutations: *KRAS* G12V ($n = 5$), *KRAS* G12D ($n = 8$), and wild-type *KRAS* or rare *KRAS* mutations ($n = 10$). Cum, cumulative; wt, wild type.