HHS Public Access

Author manuscript

Hum Pathol. Author manuscript; available in PMC 2018 October 01.

Published in final edited form as:

Hum Pathol. 2017 October ; 68: 87–91. doi:10.1016/j.humpath.2017.08.021.

Mutation of NRAS is a Rare Genetic Event in Ovarian Low-Grade Serous Carcinoma

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Abstract

Activating mutations involving the members of the RAS signaling pathway, including *KRAS*, *NRAS*, and *BRAF*, have been reported in ovarian low-grade serous carcinoma and its precursor lesion, serous borderline tumor (SBT). Whether additional genetic alterations in the RAS oncogene family accumulate during the progression of serous borderline tumor (SBT) to invasive low grade serous carcinoma (LGSC) remains largely unknown. While mutations of *KRAS* and *BRAF* occur at a very early stage of progression, even preceding the development of SBT, additional driving events, such as *NRAS* mutations, have been postulated to facilitate progression. In this study, we analyzed *NRAS* exon 3 mutational status in 98 cases that were diagnosed with SBT/atypical proliferative serous tumor (SBT/APST), non-invasive LGSC (niLGSC), or invasive LGSC (iLGSC). Of the latter, *NRAS* Q61R (CAA to CGA) mutations were detected in only 2 of 56 (3.6%) cases. The same mutation was not detected in any of the SBT/APSTs or niLGSCs. Mutational analysis for hotspots in *KRAS* and *BRAF* demonstrated a wildtype pattern of *KRAS* and *BRAF* in one of the *NRAS*-mutated cases. Interestingly, another LGSC case with *NRAS* mutation harbored a concurrent *BRAF* V600L mutation. These findings indicate that, although recurrent *NRAS* mutations are present, their low prevalence indicates that *NRAS* plays a limited role in the development of LGSC. Further studies to identify other oncogenic drivers of LGSC progression is warranted.

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Conflict of interest: The authors have no competing interests to declare.

Keywords

NRAS; Mutation; Low grade serous carcinoma

Introduction

Ovarian serous carcinoma has been classified as low- and high-grade based on distinct clinicopathological and molecular features [1]. Serous tubal intraepithelial carcinoma (STIC) is the presumable precursor lesion of high grade serous carcinoma (HGSC) and frequently harbors TP53 somatic mutations [2-4]. Most ovarian low grade serous carcinomas (LGSCs), in contrast, arise from a morphologically distinct precursor lesion, serous borderline tumor/atypical proliferative serous tumor (SBT/APST) [4-6]. There is a small subset of SBT/APSTs with micropapillary architecture that have a poor outcome; these are designated “noninvasive low-grade serous carcinoma (niLGSC)” [7]. Unlike invasive LGSC (iLGSC), niLGSC does not show a destructive growth pattern. The mechanisms by which a precursor lesion gives rise to invasive LGSC remain largely unknown. Targeted genetic analyses of candidate genes have detected somatic mutations in *KRAS* and *BRAF* in approximately 20-50% of SBT/APSTs and LGSCs (non-invasive and invasive) [8-12]. These mutations occur at a very early, pre-transformed stage, such as in cystadenoma, preceding the development of SBT/APST [13, 14]. Accordingly, additional genetic alterations other than *KRAS* and *BRAF* may accumulate as a driving force during the progression of SBT/APST to invasive LGSC.

It has been demonstrated that hemizygous or homozygous deletions of ch1p36 and ch9p21 are associated with the development of LGSC [15]. Moreover, deletions of both chromosomal regions occur more frequently in LGSCs than in SBT/APSTs [15]. Interestingly, our previous whole exome sequencing study showed that the mutation accumulation rate in bulk LGSCs was much lower than that in most adult tumors[16], consistent with the “indolent” nature of LGSCs and suggesting a low replication rate in this unique tumor type. Indeed, genome-wide analyses demonstrated very few recurrent somatic mutations in LGSCs [16].

More recently, recurrent *NRAS* mutations were reported in LGSCs, indicating an oncogenic driving role of *NRAS* in the development of LGSCs [17]. Consistent with this finding, another study identified several potential markers of progression and candidate driver genes of LGSC including *NRAS*, *USP9X*, and *EIF1AX* [18]. In this recent report, *NRAS* mutations were present in 26.3% (5/19) of LGSCs but were absent in SBT, and *NRAS* was thought to be a potent driver of LGSC tumorigenesis [18]. To further explore the role of *NRAS* in the development of LGSC, we investigated the mutational status of *NRAS* among our existing patient cohorts obtained from the Johns Hopkins Hospital and the Denmark National Patient Registry.

Materials and Methods

Selection of cases

A total of 98 cases were analyzed in this study, including 28 cases of SBT/APST, 14 cases of niLGSC, and 56 cases of iLGSC. Among these, 52 cases were obtained from the pathology archives at the Johns Hopkins Hospital, consisting of 15 SBT/APSTs, 10 niLGSCs, and 27 iLGSCs. The remaining cases (13 APSTs, 4 niLGSCs, and 29 iLGSCs) were obtained from the archives of the nationwide Denmark Pathology Data Bank [19, 20]. Histologic sections of these cases were reviewed by at least two pathologists (I.M.S./R.V. or I.M.S./D.X.). The study was approved by the Danish Data Protection Agency, the Danish Scientific Ethical Committee, and the Institutional Review Board at the Johns Hopkins Hospital.

DNA extraction and mutational analysis

Paraffin-embedded tumor tissues, identified by H&E staining of adjacent sections (tumor elements account for more than 70% of section area), were macrodissected, and genomic DNA was extracted using a QIAamp DNA FFPE Tissue Kit with an adapted protocol (Qiagen, Valencia, CA, USA). Briefly, slides bearing paraffin embedded tissue were baked at 68°C for 20 to 30 seconds; the tissue was deparaffinized 3 times with xylene, and residual xylene was removed by washing through serial dilutions of ethanol. Tumor tissue was separated from adjacent normal tissue and placed in a tube allowing for complete evaporation of residual ethanol. The tissue pellet was resuspended in Buffer ATL with added proteinase K. The rest of the procedure followed the manufacturer's instruction.

Mutational analysis was performed using conventional Sanger sequencing techniques at the *NRAS* mutational hotspot region on exon 3, including codon 61. The cases were also tested for other hotspot mutations of *KRAS* at exon 2, including codons 12-13, and *BRAF* at exon 15, including codon 600 as previously described [10, 21]. Briefly, PCR amplification was performed using genomic DNA from macro-dissected FFPE tissue with the following primers. For exon 3 of *NRAS*: forward 5′-CCCCCTTACCCTCCACAC-3′ and reverse 5′-TGGCAAATACACAGAGGAAGC-3′; for exon 15 of *BRAF*: forward 5′-TGCTTGCTCTGATAGGAAAATGA-3′ and reverse 5′-CCACAAAATGGATCCAGACAAC-3′; for exon 2 of *KRAS*: forward 5′-TAAGGCCTGCTGAAAATGACTG-3′ and reverse 5′-TGGTCCTGCACCAGTAATATGC-3′. Amplified PCR products were sequenced at Beckman Coulter, Inc., (Danvers, MA, USA), and analyzed using Mutation Surveyor DNA Variant Analysis Software.

Results

Representative histologic images of SBT/APST, niLGSC and iLGSC were present in Figure 1. Clinical factor analysis established that there was a similar age distribution between the cohort from Johns Hopkins Hospital and the cohort from the Denmark National Patient Registry. The age of patients with SBT/APSTs from the pathology files of the Johns Hopkins Hospital ranged from 24 to 67 years old (median = 43) (Table 1). The age of niLGSC patients ranged from 23 to 57 years (median = 32.5), and the age of patients with

iLGSCs ranged from 31 to 72 years (median = 50). The median ages for patients from the Denmark cohort with SBT/APSTs, niLGSCs, and iLGSCs were 43, 37, and 49.5 years, respectively. Of the 98 patients, 92 (93.8%) were of Caucasian background.

NRAS mutational status was analyzed at the hotspot region of exon 3 in 28 SBT/APSTs, 14 niLGSCs, and 56 iLGSCs from both the Johns Hopkins Hospital and Denmark cohorts (Table 1). *NRAS* Q61R (CCA to CGA) mutations were detected in 2 of 27 (7.4%) Johns Hopkins Hospital invasive LGSC cases, with none were detected in the Denmark cohort (Figure 2). The overall mutation rate of detection of *NRAS* mutation was 3.6% (2 of 56 cases). The same hotspot mutation was not detected in any SBT/APSTs or niLGSCs. In one of the two *NRAS*-mutated cases, analysis of *KRAS* and *BRAF* hotspot mutations identified no alterations. Interestingly, while all *KRAS* and *BRAF* mutations were mutually exclusive, one *NRAS*-mutated iLGSC case harbored a concurrent *BRAF* V600L mutation. Among all patients, *KRAS* mutations were detected in 15 of 28 (53.6%) SBT/APSTs, 2 of 14 (14.3%) niLGSCs, and 16 of 56 (28.6%) iLGSCs. BRAF mutations were detected in 8 of 28 (28.6%) SBT/APSTs, 2 of 14 (14.3%) niLGSCs, and 10 of 56 (17.9%) iLGSCs. Rare mutations of *BRAF* T599I and *BRAF* D594G were detected in 1 niLGSC and 1 SBT/APST, respectively (Table 1). The combined frequency of *KRAS* and *BRAF* mutations in SBT/APSTs in the Johns Hopkins Hospital cohort appeared higher than that reported previously (Table 1), likely due to selection bias with limited case number (15 cases).

Discussion

In this study, we sought to investigate the mutational profile of *NRAS* in SBT/APSTs, non-invasive LGSCs, and invasive LGSCs. Similar to previous findings, our study, based on a large retrospective cohort, did not identify *NRAS* mutations in either SBT/APSTs or non-invasive LGSCs [17, 18]. We observed a relatively low mutation frequency (3.6%) of *NRAS* in invasive LGSCs. Our data suggests that NRAS may play a very limited role in promoting aggressive behavior of LGSCs.

It is well established that LGSCs develop from SBT/APSTs in a step-wise fashion. Molecular genetic studies have highlighted the significance of the MAPK signaling pathway in the pathogenesis of LGSCs, illustrated by the fact that activating mutations in codon 12 and codon 13 of *KRAS* or in codon 600 of BRAF occur in approximately half to two-thirds of SBT/APSTs and LGSCs [1, 6, 22]. Mutations of *ERBB2*, another key factor of the Ras/Raf/MEK/MAPK signaling pathway, occur in 6% of these tumors [23]. Identical *KRAS* or *BRAF* mutations have been detected in adjacent cystadenoma epithelium, the precursor lesion of SBT/APSTs, indicating that mutations of *KRAS* and *BRAF* are early genetic events associated with initiation of low-grade serous neoplasms, and that the small subset of serous cystadenomas that acquire these mutations may progress to SBT/APST [13, 14]. Our recent study also demonstrated that a substantial proportion of LGSCs most likely represent direct progression from SBTs, as illustrated by the detection of identical *KRAS* and *BRAF* mutations in both SBT/APSTs and in subsequent LGSCs of the same patient (in preparation). This observation implies that genetic alterations, in addition to *KRAS* and *BRAF* mutations, are required to drive the progression of SBT/APSTs to invasive LGSCs.

Progression of SBT/APSTs to LGSCs is thought to be associated with novel and distinct molecular events. Two studies demonstrated that *NRAS* is a critical oncogenic driver in the development of LGSCs. In one study, the frequency of activating *NRAS* mutations was 5 of 58 (9%) in invasive tumors with adjacent borderline tumor [17]. Given that a portion of these tumors had *TP53* mutations that were consistent with HGSCs rather than LGSCs, the actual rate of *NRAS* mutation was higher. Whereas *BRAF* and *KRAS* mutations were found in both SBT/APSTs and invasive tumors, *NRAS* mutations were not found in any pure, non-ambiguous SBT/APST tumors tested. Another study demonstrated that *NRAS* mutations were detected in 26.3% of LGSCs, but none were detected in the SBT/APST cohort [18]. Consistent with previous findings, we did not observe *NRAS* mutations in either SBT/APSTs or niLGSCs. Mutations of *NRAS* Q61R were detected in 2 of 27 (7.4%) LGSCs in the Johns Hopkins Hospital cohort, and none were detected in the Danish cohort, a frequency lower than that of the previous study [18]. The precise reason for this difference is unclear. The possible explanations include selection bias due to limited sample sizes, geographic differences, and ethnic heterogeneity. Future studies with larger patient groups would provide more accurate information regarding *NRAS* mutation rates. Nonetheless, the overall mutation rate of *NRAS* appears to be low in LGSC patients. This result suggests that mutation of *NRAS* may be acquired at later stages in LGSCs, and that it may be involved in progression in a subset of tumors. It should be noted that only hotspot mutation in *NRAS* has been examined in this study. Mutation frequency of Ras/Raf/MEK/MAPK signaling pathway including *NRAS* could be underestimated due to incomplete gene sequencing.

Concurrent existence of *NRAS* and *BRAF* mutations in LGSC is unusual and contrasts with the mutually exclusive pattern of mutations among MAPK pathway components. These findings further suggest that, unlike *KRAS* or *BRAF*, *NRAS* mutations may function in a different context, such as in promoting invasiveness and aggressiveness of advanced tumors. [24-26].

In addition to mutations in genes of MAPK pathways, exome sequencing has identified mutations in novel candidate genes *EIF1AX* and *USP9X*, which are more common in LGSCs than in SBT/APSTs, suggesting that these genes play a vital role in the oncogenesis of LGSCs [18]. Mutations in *EIF1AX* and *USP9X* were almost exclusively identified in *BRAF/KRAS/NRAS* mutant tumors, suggesting a cooperative biological effect. Similarly, a most recent study demonstrated that recurrent mutations in *EIF1AX* significantly co-occurred with mutations in *NRAS* [27]. Since mutations involving MAPK pathways account for no more than two thirds of SBT/APST and LGSC cases, there remains a sizable proportion of cases with no known mutation. Accordingly, other genetic alterations, such as allelic deletions, loss of heterozygosity, or gene amplifications, may facilitate the development of LGSC. In fact, it has been found that frequent allelic deletions of 1p36 and 9p21 occur in LGSCs but not in APSTs [15]. The 1p36 region contains several candidate tumor genes that modulate cell cycle arrest and apoptosis. Chr9p21 harbors the *CDKN2A* locus, which encodes p16, p15, and p14/Arf. Similarly, loss of heterozygosity of 9p including *CDKN2A* has been observed in another study [18].

In summary, our study indicates that, although recurrent *NRAS* mutations are present, the low mutation rate suggests that NRAS itself plays a minor role in the development of LGSC.

Further studies to identify other oncogenic drivers in the progression of LGSC are warranted.

Acknowledgments

Funding/Support: This study was partly funded by the Richard W. TeLinde Gynecologic Pathology program in the Department of Pathology, The Johns Hopkins University School of Medicine. This study was also partly supported by DoD W81XWH-11-2-0230 (T.-L. Wang), NCI UO1 CA200469 (I.-M. Shih), and NCI 1R01CA215483 (I.-M. Shih)

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Highlights

- *NRAS* mutation is postulated to facilitate the development of low grade serous carcinoma (LGSC).
- *NRAS* hotspot (Q61R) mutation was detected in only 3.6% cases of LGSCs in this study.
- Low prevalence of mutation indicates that *NRAS* plays a limited role in the development of LGSC.

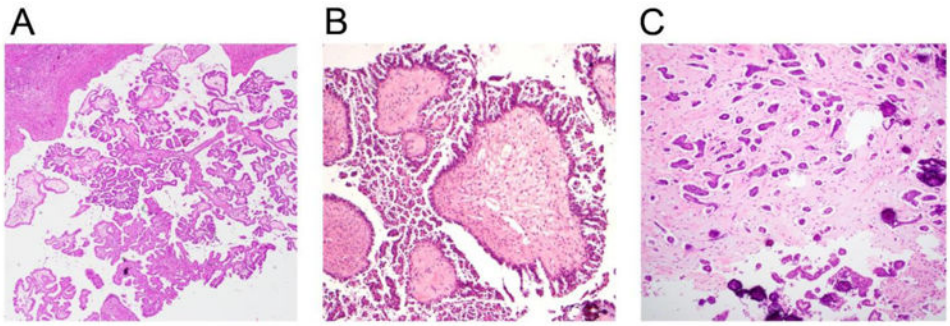

Figure 1.

Representative histologic images of three different types of ovarian serous tumor. A, Serous borderline tumor/Atypical proliferative serous tumor (SBT/APST). B, Non-invasive low grade serous carcinoma (niLGSC). C. Invasive low grade serous carcinoma (iLGSC). H&E slides, Original magnification 100×.

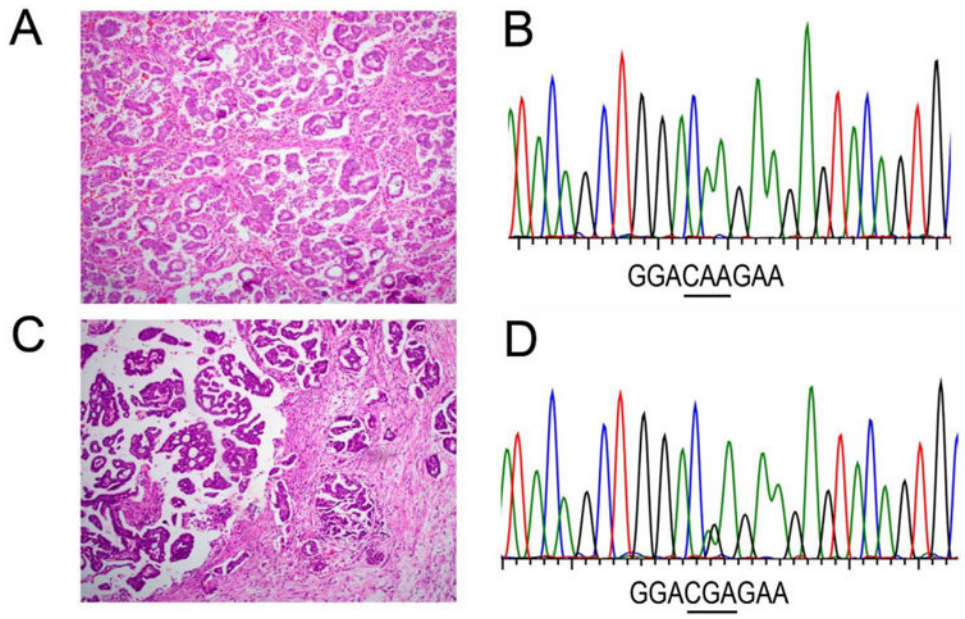

Figure 2.

Histology and nucleotide sequences of *NRAS* in two representative invasive low grade serous carcinoma cases. Case 1, A. H&E staining of tumor. B. Chromatogram of nucleotide sequence shows wild-type pattern of *NRAS* containing codon 61 in a low grade serous carcinoma. Case 2, C. H&E staining of tumor. D. Chromatogram of nucleotide sequence shows a point *NRAS* Q61R mutation (CAA to CGA) in a low grade serous carcinoma. H&E slides, Original magnification 40×.

Table 1

Mutations of *NRAS*, *KRAS*, and *BRAF* in SBT/APSTs, niLGSCs, and iLGSCs.

Pathologic Type	Patient Age		NRAS		KRAS		BRAF	
	Range	Median	Mutations	%	Mutations	%	Mutations	%
Hopkins (n=52)								
SBT/APSTs	24-67	43.0	0/15	0%	6/15 (G 12D, 4; G12V, 2)	40.0%	8/15 (V600E, 7; D594G, 1)	53.3%
niLGSCs	23-57	32.5	0/10	0%	1/10 (G12V, 1)	10.0%	2/10 (V600E, 1; T599I, 1)	20.0%
iLGSCs	31-72	50.0	2/27 (Q61R, 2)	7.4%	4/27 (G12D, 3; G12V, 1)	14.8%	7/27 (V600E, 6; V600L, 1)	25.9%
Denmark (n=46)								
SBT/APSTs	23-70	43.0	0/13	0%	9/13 (G12D, 5; G12V, 3; G12A, 1)	69.2%	0/13	0%
niLGSCs	30-54	37.0	0/4	0%	1/4 (G12D, 1)	25.0%	0/4	0%
iLGSCs	25-84	49.5	0/29	0%	12/29 (G12D, 8; G12V, 3; G12A, 1)	41.4%	3/29 (V600E, 3)	10.3%

Note: SBT/APSTs, serous borderline tumor/atypical proliferative serous tumors; niLGSCs, non-invasive low grade serous carcinomas; iLGSCs, invasive low grade serous carcinomas.