The Tyrosine Kinase Adaptor Protein FRS2 Is Oncogenic and Amplified in High-Grade Serous Ovarian Cancer

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Abstract

High-grade serous ovarian cancers (HGSOC) are characterized by widespread recurrent regions of copy-number gain and loss. Here, we interrogated 50 genes that are recurrently amplified in HGSOC and essential for cancer proliferation and survival in ovarian cancer cell lines. FRS2 is one of the 50 genes located on chromosomal region 12q15 that is focally amplified in 12.5% of HGSOC. We found that FRS2-amplified cancer cell lines are dependent on FRS2 expression, and that FRS2 overexpression in immortalized human cell lines conferred the ability to grow in an anchorage-independent manner and as tumors in immunodeficient mice. FRS2, an adaptor protein in the FGFR pathway, induces downstream activation of the Ras–MAPK pathway. These observations identify FRS2 as an oncogene in a subset of HGSOC that harbor FRS2 amplifications.

Implications: These studies identify FRS2 as an amplified oncogene in a subset of HGSOC. FRS2 expression is essential to ovarian cancer cells that harbor 12q15 amplification. Mol Cancer Res 13(3): 502–9. ©2014 AACR.

Introduction

Ovarian cancer is the second most common gynecologic malignancy and the most common cause of gynecologic cancer–related death in the United States (1). Histologically, ovarian epithelial carcinomas can be divided into high-grade serous, low-grade serous, endometroid, mucinous, and clear cell types. Clinically, high-grade serous ovarian cancer (HGSOC) accounts for 70% to 80% of all ovarian carcinomas and is characterized by its de novo invasive nature and initial sensitivity to platinum treatment. The molecular features of HGSOC include BRCA1/2 and TP53 mutations and widespread DNA copy-number alterations (2). The lack of readily targetable mutations found in HGSOC has contributed to slow progress in developing molecularly targeted therapies for this subset of ovarian cancers.

To catalog the molecular aberrations present in HGSOC, The Cancer Genome Atlas (TCGA) network performed a large-scale, multiplatform genomic profiling study of HGSOC (2). Analysis of 489 HGSOC primary tumors identified large number of recurrent somatic copy-number alterations that include 31 focal amplifications. These amplified regions encode 1,825 genes, including known oncogenes such as CCNE1 and MYC. However, the driver genes in the majority of the recurrently amplified regions remain unidentified.

In parallel to these genome characterization efforts, we initiated Project Achilles, a systematic effort to identify cancer dependencies at genome scale (3, 4). Here, by combining the output of ovarian cancer genome analysis with Project Achilles, we systematically interrogated 1,825 recurrently amplified genes in ovarian cancer to identify genes that are essential in ovarian cancer cell lines that harbor such amplifications and identified FRS2 as an amplified and essential gene in HGSOC.

Materials and Methods

Analysis of TCGA primary tumor data

Regions of copy-number amplification identified by Genomic Identification of Significant Targets in Cancer (GISTIC) analyses were used from the TCGA study on HGSOC (2). All RefSeq genes within these regions of amplification (n = 1,825) were identified and cross-referenced with genes interrogated in the Achilles screening library (n = 582). All primary HGSOC data were downloaded from the TCGA portal (http://tcga-data.nci.nih.gov/tcga). Genomic characterization data were visualized using the Integrative Genome Browser (http://www.broadinstitute.org/igv). Mutual exclusivity analysis was performed using the CBio Portal for Cancer Genomics (3, 6), which uses different thresholds for scoring regions of copy-number alteration.
Analysis of shRNA screening data

Data from genome-scale loss-of-function screening were processed as described previously (3). Briefly, 54,000 shRNAs were lentivirally delivered to 102 cancer cell lines, and the degree of representation of each shRNA in the final cell population was measured by custom Affymetrix array. Normalization, variance stabilization, and expression score calculation were conducted as specified in the modified dCHIP method (4). Scores were median-adjusted per cell line. Ovarian-specific gene dependencies were determined with three complementary methods: (i) 150 best single shRNA or (ii) 300 second best shRNA or (iii) composite of all shRNAs for the gene using KS statistics. Genes (582; 5.2%) were selected from the union of best shRNA or (iii) composite of all shRNAs for the gene using

Cell culture and generation of stable cell lines

All human cancer cell lines were cultured in previously described media supplemented with 10% FBS (Sigma; ref. 3). Immortalized human ovarian surface epithelial cells (IOSE; ref. 7) were maintained in 1:1 medium 199: DMEM supplemented with 10% FBS. CAL120, COV44, COV362, and CAOV3 cells were cultured in DMEM (Invitrogen) with 10% FBS. NIH/3T3 cells were cultured in RPMI-1640 medium (Invitrogen) with 10% FBS. HCC1143 and EFO21 cells were cultured in DMEM with 10% bovine calf serum. Lentiviruses were produced by transfection of 293T packaging cells with a three-plasmid system. To generate stable cell lines, cells were seeded into 6-well dishes for 24 hours before infection with 0.3 mL of lentivirus producing medium (Invitrogen). After the incubation, medium was replaced with fresh medium for another 24 hours before selection in media containing 2 μg/mL of puromycin or 10 μg/mL of blasticidin until the control cells were no longer viable.

Plasmids

Human FRS2 from the CCSB human ORFeome collection (8) was cloned into pLent6.3-blast (BamHI and BsrGI sites). The pLX304–LacZ was used as a control vector. The human MEKD218, D222 (or MEKDD) fragment was removed from pBabe-puro-MEKDD plasmid (9) with BamHI and SalI and inserted into pLX304–blasticidin. Lentiviral pLKO.1-puro-shRNA constructs were obtained from The Rnai Consortium or designed by custom oligo synthesis (IDT). The shRNA constructs used are as follows: control shRNA targeting LacZ (TRCN00000231710), FRS2-specific shRNAs (shFRS2#1: TRCN00000370440, shFRS2#2: 5'-CTCTAATGCTAATGATATAA-3'), shFRS2#3: 5'-TGACGTTTCGACAGTCAGCG-3').

Cell proliferation assay

CAL120, COV44, HCC1143, EFO21, COV362, and COV362a cells (3 × 10^3) were seeded into each well of 96-well plates 24 hours before infection. Six replicate infections were performed for control shRNAs and each gene-specific shRNA in the presence of 8 μg/mL polybrene for 24 hours followed by selection with 2 μg/mL of puromycin. The APT content was measured at 6 days after infection by using CellTiter-Glo luminescent cell viability assay (Promega).

Anchorage-independent growth assay

Growth in soft agar was determined by plating 5 × 10^4 cells in triplicate in 4 mL of medium containing 0.35% Noble agar (BD Biosciences), which was placed on top of 4 mL of solidified 0.6% agar. Unstained colonies greater than 100 μm in diameter were counted 4 weeks after plating using Cell Profiler software (10).

Immunoblotting

Cell lysates were prepared by scraping cells in lysis buffer (50 mmol/L Tris HCl (pH 8), 150 mmol/L NaCl, 0.5% sodium deoxycholate, and 0.1% SDS) containing complete protease inhibitors (Roche) and phosphatase inhibitors (10 mmol/L sodium fluoride and 5 mmol/L sodium orthovanadate). Protein concentration was measured by using the BCA Protein Assay Kit (Pierce). An equal amount of protein (20 μg) was separated by NuPAGE Novex Bis-Tris 4% to 12% gradient gels (Invitrogen), and then transferred onto a polyvinylidene difluoride membrane (Amersham). Antibodies against FRS2 (sc-8318) were purchased from Santa Cruz Biotechnology. Antibodies for PARP (#9532), phospho-ERK1/2 (#9101), ERK1/2(#9102) were purchased from Cell Signaling Technology and antibody specific for β-actin was obtained from Santa Cruz Biotechnology (sc-8432-HRP).

After incubation with the appropriate horseradish peroxidase–linked secondary antibodies (Bio-Rad), signals were visualized by enhanced chemiluminescence plus Western blotting detection reagents (Amersham). Alternatively, membrane was incubated with IRDye fluorescent secondary antibodies (LI-COR) and visualized by Odyssey quantitative fluorescence imaging system (LI-COR).
Tumorigenicity assay
Female NCR/nude mice (Charles River Laboratories) were obtained at 6 weeks of age. All animal experiments were approved by the Dana-Farber Institutional Animal Care and Use Committee. Tumor xenograft experiments were performed as described previously (9). NIH/3T3 cells expressing indicated constructs were trypsinized and collected in fresh media. Cells were washed and resuspended in PBS at 10^6 cells per 100 µL. Cells were injected s.c. on left and right flanks, and upper back. Two mice were used for each experimental condition. A total of 2 × 10^6 cells were injected per site, three sites per mouse. Tumor injection sites were monitored for 3 months for tumor formation. Mice were euthanized when the largest tumor on mouse reached 2 cm in largest dimension. We attempted tumor experiments with HA1E-A cells, but encountered a high background in control cells.

Statistical analysis
Unless otherwise indicated, one-way ANOVA was used (GraphPad). A P value of <0.05 was considered statistically significant. The Fisher exact test was used for tumor formation assays and mutual exclusivity analysis. A two-tailed Student t test was used for pairwise comparisons. A log-rank test was performed for animal survival studies.

Results
Identification of FRS2 as an amplified and essential gene in ovarian cancer
HGSCs are characterized by high frequency, recurrent regions of copy-number gain and loss. Recent genome-scale effort to characterize structural alterations in HGSC has identified 31 recurrently amplified chromosomal regions containing total of 1,825 genes (2). To systematically study previously unknown lineage-specific dependencies, we initiated a genome-scale effort (Project Achilles) to identify genes essential for proliferation/survival of a large number of well-characterized cancer cell lines using loss-of-function genetics with shRNAs (ref. 4). Although recent studies suggest that established ovarian cancer cell lines do not fully recapitulate the genetic alterations found in high-grade ovarian cancers (11, 12), here we have focused on those alterations found by the TCGA in human cancers and shared by these ovarian cancer cell lines. Using data from 102 cell lines of which 25 were from the ovarian lineage, we identified 582 ovarian lineage-specific gene dependencies (3). By looking at the intersection of genes involved in regions of recurrent copy number and essential in ovarian cancer cell lines, we identified 50 genes (Fig. 1A; Supplementary Table S1). Two of the 50 genes were previously identified as ovarian specific oncogenes (PAX8 and CCNE1) with similar analytic approach (3, 13).

Among the remaining genes, we focused on fibroblast growth receptor substrate 2 (FRS2) because FRS2 is (i) an adaptor protein in the FGFR pathway, (ii) is located on chromosomal region 12q15, which is focally amplified in 12.5% of 539 primary HGSCs characterized by TCGA (Fig. 1B), and (iii) was among the top 100 genes that scored by our analysis of Project Achilles and copy-number data in HGSC. We also found a structurally similar chromosomal region amplification in other cancer types such as breast invasive carcinoma, lung adenocarcinoma, lung squamous cell carcinoma, head and neck squamous cell carcinoma, gastric adenocarcinoma, and bladder urothelial carcinoma. We used Genomic Identification of Significant Targets in Cancer Version 2.0 (GISTIC 2.0) algorithm to identify the peak of amplification, which corresponds to the highest level of copy-number gain. In ovarian cancer samples, we observed the overlap between the peak of amplification and the location of the FRS2 gene. Furthermore, the focal amplification of 12q15 region in HGSC is correlated with increased mRNA expression of FRS2, suggesting the functional relevance of the copy-number gain (Fig. 1C). In addition, we also observed frequent amplification of FGFR family of tyrosine kinase receptor genes in HGSC. Strikingly, using the cBio portal, we found that HGSC samples that harbor 12q15 amplifications were often mutually exclusive with HGSC that harbor FGFR1, FGFR2, FGFR3, and FGFR4 amplifications (Fisher exact test P = 0.028; Fig. 1D). This pattern of mutations is observed in commonly mutated genes in the same pathway, such as KRAS and EGFR mutations or TP53 and MDM2 mutations. These observations implicate FGFR signaling through amplifications of FGFRs and FRS2 as a common event in HGSCs.

FRS2 is essential in cancer cell lines that harbor 12q15 amplification
To confirm that FRS2 was essential in FRS2-amplified cancer cell lines, we used two independent shRNAs to suppress FRS2 expression in three cell lines with 12q15 amplification (CAL120, -BREAST, COV644, OVARY, HCC1143, BREAST) and three cancer cell lines that contain normal copies of 12q15 (CAOV3, OVARY, EFO21, OVARY, COV362, OVARY). We used both breast and ovarian cancer cell lines because we found focal amplification of 12q15 in a large subset of the primary breast cancers (Fig. 1B). Copy-number data for these cell lines were obtained from the Broad Institute/Novartis Cancer Cell Line Encyclopedia (Fig. 2A; ref. 14). We found that FRS2 suppression by two independent shRNAs significantly decreased the proliferation of cancer cell lines that harbor the 12q15 amplification, when compared with cells that exhibit diploid copy number at 12q15 or cells infected with control shRNA (Fig. 2B). The degree of FRS2 suppression in 12q15-amplified cell lines was validated by quantitative real-time PCR (Fig. 2C). To demonstrate that FRS2 suppression induced apoptotic cell death in 12q15-amplified cell lines, we interrogated PARP cleavage after suppression of FRS2. We found increased level of cleaved PARP in 12q15-amplified cell lines compared with cell lines without 12q15 amplification (Fig. 2D). Together, these findings demonstrate that cancer cells that harbor 12q15 amplification require FRS2 expression for proliferation and survival.

FRS2 induces oncogenic transformation
To determine whether FRS2 can contribute to tumorigenesis by inducing transformation, we performed anchorage-independent growth assays and tumor xenograft experiments. In our prior studies, we have shown that human kidney epithelial cells are immortalized by coexpression of the human catalytic subunit of telomerase (hTERT) and the SV40 Early Region (HAE1 cell), and the expression of oncogenic alleles of RAS confers the ability to grow in anchorage-independent manner (15). We had previously demonstrated that the RAS oncogene can be replaced by combination of downstream effectors of the RAS signaling pathway, such as constitutively activated MEK1 (MEK-DD) and AKT1 (myristoylated AKT; ref. 9). In addition, we used the same genetic
elements to IOSE cells and fallopian tube epithelial cells and used this cell line to identify ovarian cancer oncogenes such as ID4 (16). We note that recent reports suggest that both fallopian tube and ovarian surface epithelial cells can serve as the origin for HGSOC and have not noted differences in the transformation potential of cells from either lineage (16–20).

As previous studies have shown that FRS2 preferentially activates the MAPK pathway, we overexpressed FRS2 in HA1E cell lines expressing constitutively active myristoylated AKT (HA1E-A) to determine whether FRS2-mediated MAPK pathway activation complemented AKT pathway activation to induce transformation. We measured anchorage-independent growth with FRS2 overexpression and found that FRS2 overexpression was sufficient to induce anchorage-independent colony formation of HA1E-A cells compared with cells expressing the control LacZ (Fig. 3A). The number of colonies formed with FRS2 overexpression is significantly higher (P < 0.001) compared with constitutively activated MEK, suggesting possible activation of additional pathways that contribute to the transformation process. We also conducted the same experiment in IOSE cells to show that FRS2 also induced transformation in ovarian epithelial cells (Fig. 3B).

Next, we determined whether expression of FRS2 also induced tumor formation in vivo by expressing FRS2 in NIH3T3 mouse fibroblast cells and implanting these cells subcutaneously in immunodeficient mice. At 11 weeks, we observed that tumors formed in 33% (2 of 6) of the injection sites harboring cells expressing FRS2, but failed to observe any tumors in sites harboring control cells (Fig. 3C). We note that because we implanted tumors in several sites in each mouse, and we terminated the

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**Figure 1.**

Amplification and overexpression of FRS2 in primary HGSOCs and ovarian cancer cell lines. A, FRS2 is one of the 50 genes that are recurrently amplified in primary ovarian tumors and essential for ovarian cancer cell proliferation and survival. B, copy-number profile along chromosome 12q of human tumor samples. FRS2 was amplified in multiple cancer types, including ovarian, breast, lung squamous, lung adenocarcinoma, stomach, head and neck (H&N), and bladder. Each vertical line represents one tumor sample. Red, copy-number gain; blue, copy-number loss. C, level of FRS2 mRNA expression in primary tumors correlates with the copy number. Copy number is divided into four categories based on log2 of copy numbers. “Amplification” is defined as log2 (copy number) more than 1; “gain” is between 0.2 and 1; “normal” is between −0.2 and 0.2; “loss” is less than −0.2. D, FRS2 amplification and FGFR1, FGFR2, FGFR3, and FGFR4 amplifications are mutually exclusive in HGSOCs. Data were analyzed using the cBio portal rather than GISTIC.
experiment before observing tumor growth in all sites, these experiments may underestimate the tumorigenicity of these cells. These observations confirm that FRS2 overexpression can induce oncogenic transformation in human kidney fibroblasts or mouse fibroblasts by promoting anchorage-independent growth or in vivo xenograft tumor formation.

**Figure 2.** Suppression of FRS2 decreases the proliferation of ovarian and breast cancer cells harboring 12q15 amplification. A, SNP array colorgram showing genomic amplification of chromosome 12q15 in ovarian and breast cancer cell lines. Red, copy-number amplification; blue, copy-number deletion. B, consequences of FRS2 suppression on the proliferation of cancer cell line that either harbor 12q15 amplification (CAL120, HCC1143, and COV644) or normal copy number of 12q15 (CAOV3, COV362, and EFO21) normalized to cells treated with shLacZ. Red, cell lines treated shFRS2 #1; black, cell lines treated with shFRS2#2; **, P < 0.01 compared with control shLacZ, the Student t test was used. C, quantitative RT-PCR of FRS2 expression in FRS2-amplified (red) and -nonamplified (black) cell lines. D, increased apoptosis in FRS2-amplified cell lines (red) upon FRS2 suppression, shown by increased PARP cleavage.
FRS2 Is an Ovarian Cancer Oncogene

Discussion

Here, we identified FRS2 as one of the 50 genes that are recurrently amplified in HGSOCs and essential to survival in ovarian cancer cell lines. FRS2 belongs to the 12q15 genomic region that is focally amplified in 12.5% of HGSOC. Using independent shRNAs targeting against FRS2, we showed the expression of FRS2 was essential for survival in cancer cells with 12q15 amplification. We also discovered that overexpression of FRS2 in immortalized kidney fibroblast or ovarian epithelial cells promoted anchorage-independent growth and tumorigenesis in mice. Together, these observations nominate FRS2 as an amplified oncogene in a subset of HGSOCs.

In addition to HGSOC, 12q15 amplification containing FRS2 is found in other cancer types. 12q15 amplification containing FRS2 is focally amplified in 9.2% of breast invasive carcinomas. Indeed, we found that breast cancer cell lines that harbor 12q15 amplification are also sensitive to suppression of FRS2. Furthermore, new evidence has suggested the oncogenic role of FRS2 and 12q15 amplification in high-grade liposarcomas through whole-exome sequencing and demonstrated sensitivity of FRS2-amplified high-grade liposarcoma cell lines to FRS2 suppression through shRNAs (23, 24). These studies support FRS2 as a bona fide oncogene in a variety of cancers and a potential therapeutic target for a subset of cancers that harbor such amplification.

The discovery of FRS2 as an amplified oncogene adds to the family of FGFR signaling components that are critical to tumorigenesis in many cancer types. For example, mutations or amplifications of multiple FGFR have been reported in bladder cancer (25), gastric cancer (26), endometrial cancer (27), and non–small cell lung cancer (NSCLC; refs. 28, 29). Large-scale genome-wide association studies have also linked breast cancer risk loci to FGFR2 (30). Moreover, FGF ligands may also contribute to cancer or therapy resistance as evidenced by FGF19 amplifications in liver cancer (31) and the observed therapeutic effect of neutralizing anti-FGF antibodies (32).

The 12q15 genomic region contains 15 genes and FRS2 resides at the peak of this amplicon (Supplementary Table S2). Prior work in high-grade liposarcoma, which exhibits a broader region of amplification (12q13-12q15) than HGSOC, suggested that in addition to FRS2, other genes such as CDK4 and MDM2 may contribute to cell transformation (23). Although neither CDK4 nor MDM2 is located within the 12q15-amplified region in HGSOC, this finding does not preclude the possibility that other genes in the genomic region may cooperate to drive various stages of tumorigenesis. Indeed, we recently demonstrated that multiple genes resident in a recurrently amplified region (3q26) contribute to cell transformation by inducing different cancer-associated phenotypes, suggesting that further studies involving other assays will be necessary to investigate the function of these other genes (33).
Here, we show a new functional class of adaptor proteins as driver oncogene in ovarian cancer. The adaptor proteins lack intrinsic enzymatic activities, but mediate protein–protein interactions that drive protein complex formation. Classic examples of adaptor proteins include GRB2 in receptor tyrosine kinase (RTK) signaling (34) and MYD88 in NF-kB signaling (35). FRS2 was originally discovered as a docking site for coordinated assembly of a multiprotein complex that includes GRB2, GAB1, and SOS1 and serves a critical role in the FGFR signaling pathway (Fig. 4A; refs. 21, 36). Amplification or overexpression of the adaptor proteins may amplify signaling downstream of receptors and may mediate resistance to RTK-targeted therapies or confer de novo sensitivity to signaling pathway inhibitors. We previously identified CRKL, an adaptor protein involved in RAS and RAP signaling, as an amplified oncogene in NSCLC (37) that also mediates resistance to an EGFR inhibitor in EGFR-mutant lung cancer cells. More recently, through a multiplexed in vivo transformation screen, we found another adaptor protein, GAB2, as an amplified ovarian cancer oncogene that activates PI3K signaling (38). Ovarian cancer cells with GAB2 alteration are sensitive to PI3K pathway inhibition. An independent analysis of TCGA datasets across 16 cancer types identified 75 amplified and potentially druggable genes, including FRS2 and EGFR family adaptors GRB2 and GRB7 (39). Together, these findings suggest that adaptor proteins such as FRS2 play key roles in cell transformation and resistance to therapy. Identifying alterations in these adaptor proteins may allow for the identification of resistant tumors and represent novel targets.

Disclosure of Potential Conflicts of Interest

W.C. Hahn is a consultant to Novartis and RRS is an employee of Astellas Pharma U.S. No potential conflicts of interest were disclosed by the other authors.

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Figure 4.
FRS2 promotes tumorigenesis via activation of the MAPK pathway. A, FRS2 functions as an adaptor protein in the FGFR signaling pathway, adapted from Turner and Grose (40). B, effect of FRS2 overexpression on phosphorylation of ERK in 293T cells and ovarian epithelial cells. C, effect of FRS2 suppression on phosphorylation of ERK in the cancer cell line with 12q15 amplification.
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