Amplification of the *NSD3-BRD4-CHD8* pathway in pelvic high-grade serous carcinomas of tubo-ovarian and endometrial origin

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Abstract. Identification of novel therapeutics in pelvic high-grade serous carcinoma (HGSC) has been hampered by a paucity of actionable point mutations in target genes. The aim of the present study was to investigate the extent of amplification of the therapeutically targetable *NSD3-CHD8-BRD4* pathway in pelvic HGSC, and to determine whether amplification is associated with worse prognosis. The Cancer Genome Atlas (TCGA) ovarian and endometrial cancer cohorts were retrospectively analyzed via online data-mining tools to test the association of *NSD3*, *CHD8* and *BRD4* genomic alterations with survival of pelvic HGSC patients. It was demonstrated that amplification of the *NSD3-CHD8-BRD4* pathway in the ovarian HGSC cohort (observed in 18% of the cases, 88/489) was significantly associated with worse overall and progression-free survival compared with non-amplified cases. In addition, amplification of *NSD3*, *CHD8* and *BRD4* also occurred in 9% (21/232) of overall endometrial cancer TCGA cases, which was associated with worse overall survival. In the endometrial cancer TCGA cohort, *NSD3*, *CHD8* and *BRD4* amplification occurred specifically in the serous carcinoma (25%, 13/53) and 'serous-like' copy number high endometrial carcinoma (33%, 20/60) subgroups, compared with the polymerase e (0%, 0/17), microsatellite instability high (0%, 0/65) or low copy number (1%, 1/90) subgroups. These findings support the hypothesis that amplification of the *NSD3-BRD4-CDH8* axis is frequent in pelvic HGSC of both ovarian and endometrial origin, and that this pathway is potentially targetable in a subset of HGSC patients.

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Introduction

Pelvic high-grade serous carcinoma (HGSC) of either tubo-ovarian or endometrial origin is an aggressive and deadly gynecological malignancy with few available targeted therapies, due in part to the absence of high-frequency oncogenic point mutations in drug target genes (1-4). Recently, The Cancer Genome Atlas (TCGA) projects have advanced our understanding of the genomic landscape of ovarian and endometrial HGSC and confirmed a prevalence of somatic tumor protein p53 mutations and extensive copy number alterations, such as numerous DNA amplifications and deletions (1,5).

Gene amplification in neoplastic cells may be a mechanism for overexpression of cancer-promoting driver genes with potential 'druggable' properties (6). In ovarian HGSC, it has been previously demonstrated that BRD4, a BET bromo-domain-containing protein that is associated with acetylated chromatin and transcriptional activation, is amplified in a small subset (12%) of cases (7-10), and *BRD4* amplification in ovarian HGSC may be associated with worse overall and progression-free survival (9). Recently, BRD4 has become an attractive target for cancer therapy, as specific small-molecule BRD4/BET inhibitors, such as JQ1 and OTX015/MK-8628, are currently in clinical trials for cancer (ClinicalTrials. gov identifier: NCT01713582) (11-13). In ovarian cancer cell lines and patient-derived xenograft model systems of HGSC, suppression of BRD4 using the small-molecule BET inhibitors JQ1 and/or I-BET151 exerted robust antitumor effects (7,10), thereby providing a rationale for further investigating genomic alterations of the BRD4 pathway and the clinical benefits of BRD4-specific inhibitors in pelvic HGSC.

In several tumor types, BRD4 has been shown to selectively regulate transcription of key oncogenic drivers, such as *CMYC* (14-17), by specifically targeting cell type-specific enhancer sequences (i.e., super enhancers) (18). In addition, the histone methyltransferase NSD3 short isoform is an adaptor that couples BRD4 to the chromatin remodeling factor CHD8 to activate transcription (19). In leukemia cells, genetic targeting of NSD3 or CHD8 mimics the effects of BRD4 inhibition. Furthermore, BRD4, NSD3 and CHD8 co-localize across the genome, and BRD4-NSD3-CHD8 protein complexes are evicted from chromatin super-enhancer regions upon chemical inhibition by BRD4 small-molecule inhibitors (19). The goal of this study was to investigate the

extent of amplification of the *NSD3-CHD8-BRD4* axis in pelvic HGSC of both tubo-ovarian and endometrial origin, and to determine whether amplification of this pathway is associated with worse prognosis and survival in pelvic HGSC patients.

Materials and methods

Study type and design. An observational retrospective secondary data analysis of clinically annotated multi-platform ovarian and endometrial cancer-omics datasets from the TCGA project was performed. Publicly available online tools were used to data mine the ovarian and endometrial carcinoma TCGA datasets and to investigate the survival of pelvic HGSC patients in association with genomic and transcriptomic alterations of *NSD3*, *BRD4* and *CHD8*.

TCGA cohorts, gene amplification and survival analysis. Clinical data from the ovarian carcinoma TCGA study (1), or the endometrial carcinoma TCGA study (5), were analyzed with respect to *NSD3*, *BRD4* and *CHD8* amplification via the cBio Cancer Genomics Portal (http://cbioportal.org; Memorial Sloan Kettering Cancer Center, New York, NY, USA) (20,21). In the present study, copy number gain was defined as gain of 1 gene copy number, while amplification was defined as gain of ≥2 gene copy numbers.

From the ovarian carcinoma TCGA study, the following cohorts of patients were queried: i) All tumors (n=557), ii) all complete tumors (n=316), iii) cases with available copy number alteration data (n=489), iv) cases with complete response to primary therapy (n=276) and v) cases that were platinum-sensitive (n=197). The cohorts were queried specifically for *NSD3* (also referred to as *WHSC1L1*), *BRD4* and *CHD8* gene amplification in the cBio portal by using the advanced Onco Query Language (*WHSC1L1*: AMP; *BRD4*: AMP; *CHD8*: AMP). Kaplan-Meier overall and disease-free survival analyses were obtained via the cBio portal, with significance estimated with the log-rank test (20,21).

A similar survival analysis via the cBio portal and via the advanced Onco Query Language was performed with the endometrial carcinoma TCGA study, in which the following cohorts were analyzed: i) Complete endometrioid-type tumors (n=232), ii) copy number high tumors (n=60), iii) copy number low tumors (n=90), iv) microsatellite instability (MSI; hypermutated) tumors (n=65), v) polymerase e (POLE; ultramutated) tumors (n=17) and vi) tumors with specific serous-type histology (n=53).

mRNA expression analysis. TCGA cohorts were queried specifically for *NSD3* (*WHSC1L1*) and *CHD8* mRNA overexpression in the cBio portal by using the advanced Onco Query Language. To identify significant *NSD3* and *CHD8* mRNA overexpression with Z scores of either +2.0 or +3.0, the following advanced Onco Query Language was used respectively (*WHSC1L1*: EXP>2; *CHD8*: EXP>2) or (*WHSC1L1*: EXP>3; *CHD8*: EXP>3). Kaplan-Meier overall and disease-free survival analysis were obtained via the cBio portal, with significance estimated using the log-rank test.

Results

Amplification of NSD3, CHD8, BRD4 and other BRD4-associated genes in ovarian HGSC. Clinical data from the ovarian carcinoma TCGA cohort, specifically 489 cases with copy number alteration data, was integrated with *NSD3*, *CHD8* and *BRD4* amplification data from the TCGA data portal via the cBio Cancer Genomics portal (http://cbioportal.org) (1,20,21). Somatic amplification of at least one member of the *NSD3-CHD8-BRD4* axis was observed in 18% (88/489) of the cases (Fig. 1A). Survival analysis of TCGA patients with ovarian HGSC (n=489), revealed that patients with somatic amplification of at least one member of the *NSD3-CHD8-BRD4* pathway had significantly worse overall and progression-free survival compared with those with non-amplified pathway members, with log-rank test P-values of 0.00078 and 0.00085, respectively (Fig. 1B and C). The median number of months until relapse was 14.0 for cases with pathway amplification, compared with 17.6 months for cases without amplification. Patients with amplification had a median survival of 35.8 months compared with a median survival of 45.0 months for patients without amplification.

Our analysis focused on the ovarian TCGA cohort of cases with copy number alterations (n=489), as this cohort maximizes the number of cases with gene amplifications. However, significant worse overall survival and progression-free survival effects (P<0.05) with *NSD3*, *BRD4* and *CHD8* amplification were also observed in the following ovarian HGSC TCGA cohorts (data not shown): i) All tumors (n=557), ii) all complete tumors (n=316), iii) platinum-sensitive tumors (n=197) and iv) tumors with complete response to primary therapy (n=276). In addition, amplification of *BRD4*-associated genes in the TCGA ovarian carcinoma cohort appeared to be specific to amplification of *NSD3*, *CHD8* and *BRD4*, since other known BRD4-associated genes were not significantly amplified in the TCGA study. For example, amplification of the following known BRD4-associated genes occurred at <1% of total cases for each gene: *CDK9*, *CCNT1*, *HEXIM1*, *MLLT1*, *AFF1*, *KAT5*, *MED12* and *MED24* (data not shown).

Co-occurrence of NSD3 and CHD8 amplification in ovarian HGSC. It has been previously demonstrated that *BRD4* amplification alone in ovarian HGSC (observed in 12% of the cases) is associated with worse survival (9). Furthermore, in the ovarian HGSC TCGA cohort, there was a significant tendency towards co-occurrence of *NSD3* and *CHD8* amplification (P<0.001, log odds ratio=2.383), while there was a trend for mutual exclusivity of *NSD3* and *BRD4* amplification (P=0.091, log odds ratio <-3). For these reasons, we assessed whether amplifications of *NSD3* and/or *CHD8* were independently associated with worse survival. Somatic amplification of *NSD3* and/or *CHD8* was observed in 7% (34/489) of the cases, and amplification of *NSD3* and/or *CHD8* was significantly associated with worse overall and progression-free survival compared with those in non-amplified cases; log-rank test P=0.0296 and P=0.0150, respectively (Fig. 2A and B). The median number of months to relapse was 12.8 for cases with *NSD3* and/or *CHD8* amplification compared with 17.5 months for cases without amplification. Patients with *NSD3* and/or *CHD8* amplification had a median survival of 33.4 months
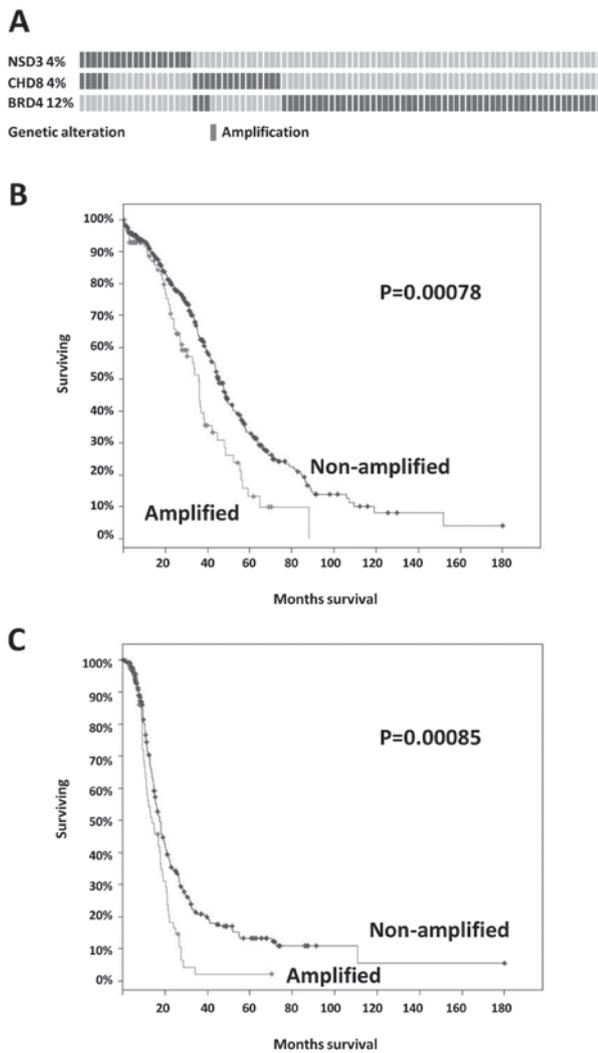
Figure 1. Amplification of the *NSD3-CHD8-BRD4* pathway and survival in ovarian HGSC. (A) Amplification of the *NSD3-CHD8-BRD4* pathway in ovarian HGSC (only tumors exhibiting amplification of at least one member of the pathway are shown). (B and C) Survival Kaplan-Meier curves for patients whose tumor exhibited amplification of at least one member of the *NSD3-CHD8-BRD4* pathway: (B) Overall and (C) disease-free survival of TCGA cases with copy number alteration data (n=489) and *NSD3*, *CHD8* and/or *BRD4* amplification. HGSC, high-grade serous carcinoma; TCGA, The Cancer Genome Atlas.

Figure 2. Survival Kaplan-Meier curves for patients with ovarian high-grade serous carcinoma with tumors exhibiting amplification of *NSD3* and/or *CHD8*. (A) Overall and (B) disease-free survival of TCGA cases with copy number alteration data (n=489), *NSD3* and/or *CHD8* amplification. TCGA, The Cancer Genome Atlas.

compared with a median overall survival of 44.5 months in patients without amplification. These results suggest that the survival effects of *NSD3/CHD8* amplification are mutually exclusive to *BRD4* amplification in ovarian HGSC.

Overexpression of NSD3 and CHD8 mRNA in ovarian HGSC. The *NSD3* and *CHD8* mRNA data for the ovarian carcinoma HGSC TCGA cohort were next examined. Similar to previously reported data for *BRD4* (9), *NSD3* and *CHD8* mRNA levels tended to increase with gene copy number increases (Fig. 3A and B). To define significant *NSD3* and *CHD8* mRNA upregulation, Z score thresholds of either +2.0 or +3.0 were used via the cBio portal, specifically in the cohort of cases with copy number alterations (n=489). By using a Z score threshold of +2.0, *NSD3* mRNA expression levels were increased in 9% (43/489) of the cases, and *CHD8* mRNA expression levels were increased in 7% (36/489) of the cases. By contrast, by

using a more stringent Z score threshold of +3.0, *NSD3* mRNA expression levels were increased in 3.5% (17/489) of the cases, and *CHD8* mRNA expression levels were increased in 2.9% (14/489) of the cases.

Using a Z score threshold of +2.0, in 78.9% of *NSD3*-amplified cases (15/19), *NSD3* amplification resulted in *NSD3* mRNA upregulation. A similar analysis revealed that, in 85% of *CHD8*-amplified cases (17/20), *CHD8* amplification resulted in *CHD8* mRNA upregulation. Using a more stringent Z score threshold of +3.0, in 57.9% of *NSD3*-amplified cases (11/19), *NSD3* amplification resulted in *NSD3* mRNA upregulation, while in 55.0% of *CHD8*-amplified cases (11/20), *CHD8* amplification resulted in *CHD8* mRNA upregulation.

mRNA overexpression of *NSD3* and/or *CHD8* was associated with worse overall survival, with Z scores of either +2.0 (log-rank test P=0.0247, Fig. 4A), or with a more stringent Z score of +3.0 (log-rank test P=0.0064, Fig. 4B). A smaller P-value with a more stringent Z score of +3.0 compared with a score of +2.0 suggests a dose-response type overall survival effect with increasing levels of *NSD3* and *CHD8* mRNA. However, there was no significant difference in progression-free survival in cases with *NSD3* and/or *CHD8* mRNA upregulation, with Z scores of either +2.0 or +3.0 (log-rank test P=0.38 for either Z score).

Amplification of NSD3, CHD8 and BRD4 in endometrial carcinoma. We next investigated the endometrial carcinoma
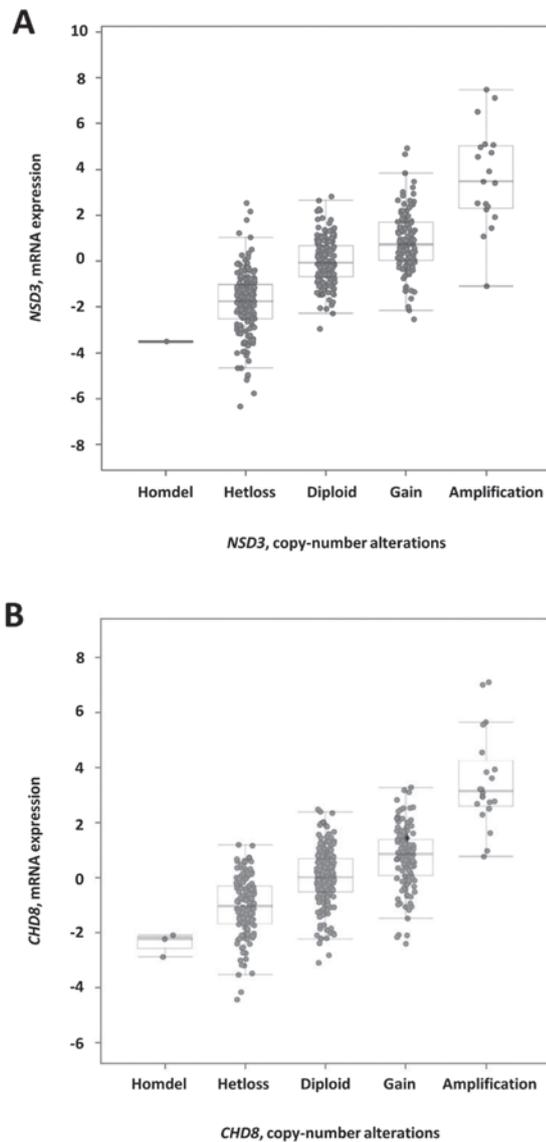
Figure 3. *NSD3* and *CHD8* mRNA expression vs. copy number data for ovarian high-grade serous carcinoma. (A) *NSD3* and (B) *CHD8* mRNA levels tended to increase with increased copy number.

Figure 4. Overall survival with *NSD3* and/or *CHD8* mRNA upregulation. (A) Kaplan-Meier curve depicting overall survival with *NSD3* and/or *CHD8* mRNA upregulation (Z score threshold of +2.0), of *NSD3* and/or *CHD8* mRNA levels in ovarian high-grade serous carcinoma. (B) Kaplan-Meier curve depicting overall survival with *NSD3* and/or *CHD8* mRNA upregulation (Z score threshold of +3.0), of *NSD3* and/or *CHD8* mRNA levels in ovarian high-grade serous carcinoma.

TGCA cohort in order to determine whether amplification of the *NSD3-CHD8-BRD4* axis occurred in endometrial carcinomas, and whether amplification in endometrial cancer was associated with specific survival outcomes and specific histological or molecular subgroups. Initial analysis of 232 endometrioid-type tumors with complete molecular data revealed amplification of at least one member of the pathway (*NSD3*, *CHD8* and/or *BRD4*), in 9% (21/232) of the overall endometrioid cancer TCGA cases (data not shown). Of note, amplification of at least one pathway member was significantly associated with worse overall patient survival; log-rank test P=0.015 (Fig. 5A), while no significant difference in progression-free survival was observed; log-rank test P=0.15 (progression-free survival curve not shown).

The TCGA endometrial endometrioid carcinoma cohort has been stratified into four different molecular subgroups, each with distinct survival outcomes: i) 'Serous-like' copy number high alterations (worst prognosis); ii) MSI hypermutated; iii) copy number low alterations; and iv) POLE ultramutated

(best prognosis) (5). For this reason, it was assessed whether amplification of the *NSD3-CHD8-BRD4* axis in endometrioid adenocarcinoma was associated with specific molecular subgroups. In the TCGA cohort, amplification of at least one member of the *NSD3-CHD8-BRD4* pathway occurred specifically in the 'serous-like' copy number high endometrial endometrioid carcinoma (33%, 20/60) subgroup (Fig. 5B), compared with the MSI high (0%, 0/65) (Fig. 5C), low copy number (1%, 1/90) (Fig. 5D) or POLE ultramutated (0%, 0/17, data not shown) molecular subgroups.

Finally, it was investigated whether amplification of the *NSD3-CHD8-BRD4* pathway also occurred in endometrial carcinomas of serous histological subtype. The TGCA study has additionally characterized the genomic landscape of 53 endometrial serous carcinomas, of which 25% (13/53) harbored *NSD3*, *CHD8* and/or *BRD4* amplification (Fig. 5E). In endometrial serous and in endometrioid 'serous-like' carcinoma, amplification of the *NSD3-CHD8-BRD4* axis was not associated with worse overall or progression-free survival (data not shown). However, the overall findings support the notion that
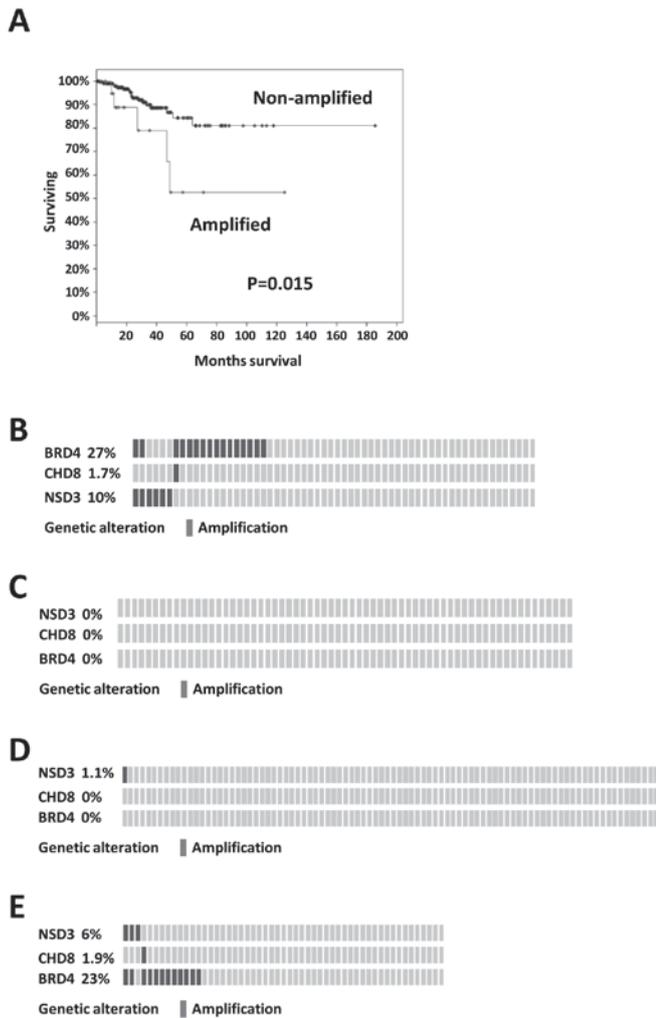
Figure 5. Amplification of the *NSD3-CHD8-BRD4* pathway in the endometrial endometrioid and serous carcinoma TCGA cohorts. (A) Overall survival Kaplan-Meier curve for patients with tumors exhibiting amplification of at least one member of the *NSD3-CHD8-BRD4* pathway, regardless of subtype. Amplification of the *NSD3-CHD8-BRD4* pathway in the (B) endometrioid 'serous-like' carcinoma, (C) endometrioid MSI high and (D) endometrioid low copy number molecular subgroups, and (E) in tumors with specific serous-type histology. TCGA, The Cancer Genome Atlas; MSI, microsatellite instability.

amplification of members of the *NSD3-BRD4-CDH8* pathway occurs frequently in a subset of pelvic HGSCs of both ovarian and endometrial origin.

Discussion

Recently, the chromatin regulators have become attractive targets for cancer therapy. BRD4 is a chromatin reader, which binds acetylated chromatin at specific promoters/enhancers and recruits additional factors to specific genomic sites. The BRD4 chromatin-remodeling complex is partly composed of BRD4-NSD3-CHD8 protein complexes, which all co-localize in the genome to promote transcription and expression of target genes such as *CMYC*, *CD274* (encoding PD-L1) or *ALDH1A1* (18,22-24). Importantly, small-molecule inhibitors of BRD4, such as JQ1 and I-BET151, have been developed and may hold promise for the treatment of cancer, including ovarian HGSC and endometrial adenocarcinoma (7,10,25).

We used the publicly available data of the TCGA project on ovarian and endometrial carcinomas to investigate the extent of genomic alterations of the NSD3-CHD8-BRD4 chromatin-remodeling complex in pelvic HGSCs. We herein propose that amplification of the *NSD3-CDH8-BRD4* axis frequently occurs in a subset of pelvic HGSC of tubo-ovarian as well as endometrial origin. In patients with tubo-ovarian HGSCs, amplification of this pathway may be associated with unfavorable prognosis and survival. In endometrial cancers, amplification occurred more specifically in endometrial serous and in endometrioid 'serous-like' carcinomas, which are intrinsically more aggressive compared with the more commonly encountered low-grade endometrioid endometrial adenocarcinoma. The percentage of cases exhibiting *NSD3-CHD8-BRD4* pathway amplification appears to be similar among ovarian HGSC (18%), endometrial serous carcinoma (25%) and endometrial serous-like carcinoma (33%), suggesting a shared mechanism of tumor progression in a subset of pelvic HGSCs. These findings support the hypothesis that the *NSD3-BRD4-CHD8* pathway is potentially targetable with the newly developed BRD4-specific small-molecule inhibitors in a subset of HGSC patients of either tubo-ovarian or endometrial origin.

NSD3, *CHD8* and *BRD4* are located in 3 different amplicons in the genome, and within these amplicons, there is evidence to suggest that *NSD3*, *CHD8* and/or *BRD4* may be putative cancer driver genes rather than passenger genes. First, amplification was identified in two different TCGA datasets of pelvic HGSCs of i) ovarian and ii) endometrial origin. Second, amplification of *NSD3*, *CHD8* and *BRD4* correlated with increased mRNA expression, and in ovarian HGSC, worse patient survival is also observed with increased *NSD3*, *CHD8* and *BRD4* mRNA levels (9). Third, the oncogenic activity of NSD3 and BRD4 in ovarian HGSC has been previously validated by inhibition of NSD3 via siRNA/shRNA knockdown or of BRD4 via BRD4-specific small-molecule inhibitors, which resulted in decreased proliferation of ovarian cancer cells (6). Similar cytotoxic results were also seen in endometrial cancer cell culture and xenograft systems, wherein the BRD4-specific inhibitor, JQ1, suppressed growth of phosphatase and tensin homolog-positive endometrial cancer cells (25). Additionally, it has been demonstrated that knockdown of NSD3 (also referred to as *WHSC1L1*) in breast cancers with amplification of the 8p11-12 region resulted in significant decreases in cell proliferation (26).

However, it should be noted that certain nearby amplified genes within the amplicons also displayed increased mRNA expression in the TCGA cohort. For example, *RAB2B* is located in the same amplicon as *CHD8*, *NOTCH3* is located in the same amplicon as *BRD4*, and *BAG4* is located in the same amplicon as *NSD3*, all of which exhibited gene amplification and increased mRNA expression in a subset of ovarian HGSC patients from the TCGA cohort (data not shown). In addition, based on our overall and progression-free survival curve analyses, prognosis appears to be better correlated with amplification rather than mRNA expression, suggesting a potential collaborative role of co-amplified genes in the amplicons. Despite being a part of amplicons, there are several studies demonstrating the importance of *NSD3*, *CHD8* and *BRD4* in oncological processes (27-31), and it is noteworthy that

amplicons may not necessarily contain only one oncogene, but may act as a unit with several genes of importance (26). Future knockdown of *NSD3*, *BRD4* and *CHD8* or treatment of HGSC displaying pathway amplification with BRD4-specific small-molecule inhibitors are required to confirm the growth-promoting properties of specific gene amplification in patients with pelvic HGSC.

Possible therapeutic downstream effector genes that are regulated by the NSD3-BRD4-CHD8 complex in pelvic HGSC include *CMYC*, *CD274* (encoding PD-L1) and/or *ALDH1A1*. In a human xenograft model, BRD4 inhibition was effective in a subset of ovarian HGSCs that exhibited high CMYC or MYCN levels (7). In another ovarian cancer mouse model, treatment with BRD4-specific inhibitors limited tumor progression via immunotherapeutic effects by significantly reducing PD-L1 expression on tumor cells, dendritic cells and macrophages, and by increasing the activity of antitumor cytotoxic T cells (23). In addition, a third independent group has demonstrated that combination treatment of ovarian cancer cells with both cisplatin and the BRD4-specific inhibitor, JQ1, suppressed the outgrowth of cisplatin-resistant cells and improved the survival of ovarian cancer-bearing mice by suppressing ALDH1A1 levels and stem cell-like characteristics (24). In the ovarian HGSC TCGA cohort, our finding that worse overall survival is also observed with increased *NSD3*, *BRD4* and *CHD8* mRNA levels, in addition to gene amplification, suggests that this survival effect may be specific to *NSD3-BRD4-CHD8* function by regulating the gene expression of potential downstream target genes.

Due to the limitations of the TCGA studies (such as relatively short follow-up time, lack of comorbidities and clinical covariables), these findings are exploratory and hypothesis-generating rather than definitive. For this reason, future independent studies with multivariate analyses and long-term follow-up to determine the effect of other clinical covariables (including age, stage, comorbidities and treatment regimens) on survival outcome are required to confirm our TCGA findings. However, it is worth highlighting that the vast majority of pelvic HGSCs from the TCGA study were high-stage (stage III or IV) as well as high-grade (1,5). In addition, our analysis also revealed significant survival effects with pathway amplification, specifically in a platinum-sensitive ovarian TCGA cohort and in an ovarian TCGA cohort with complete response to primary therapy.

Finally, we hypothesized that *NSD3-BRD4-CHD8*-amplified pelvic HGSCs may exhibit increased sensitivity to newly developed BRD4 small-molecule inhibitors. Future studies, including animal xenografts or cell culture models, are required to further elucidate the mechanisms by which amplification and overexpression of the pathway may affect tumor progression and patient survival in patients with HGSC. In summary, this study presents evidence that the *NSD3-BRD4-CHD8* pathway is amplified and overexpressed in a subset of pelvic HGSCs of both tubo-ovarian and endometrial origin, and that amplification and overexpression may be associated with worse prognosis and survival. Our findings suggest that target inhibition of the BRD4 axis with specific inhibitors should be tested in a subset of patients with pelvic HGSC of gynecological (endometrial or tubo-ovarian) origin.

Acknowledgements

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