IJC
International Journal of Cancer

Identification of novel cyclin gene fusion transcripts in endometrioid ovarian carcinomas

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Formation of fusion genes is pathogenetically crucial in many solid tumors. They are particularly characteristic of several mesenchymal tumors, but may also be found in epithelial neoplasms. Ovarian carcinomas, too, may harbor fusion genes but only few of these were found to be recurrent with a rate ranging from 0.5 to 5%. Because most attempts to find specific and recurrent fusion transcripts in ovarian carcinomas focused exclusively on high-grade serous carcinomas, the situation in the other carcinoma subgroups remains largely uninvestigated as far as fusion genes are concerned. We performed transcriptome sequencing on a series of 34 samples from ovarian tumors that included borderline, clear cell, mucinous, endometrioid, low-grade and high-grade serous carcinomas in search of fusion genes typical of these subtypes. We found a total of 24 novel fusion transcripts. The *PCMTDI-CCNL2* fusion transcript, which involves a member of the cyclin family, was found recurrently involved but only in endometrioid carcinomas (4 of 18 tumors; 22%). We also found three additional fusion transcripts involving genes belonging to the cyclin family: *ANXA5-CCNA2* and *PDE4D-CCNB1* were detected in two endometrioid carcinomas, whereas *CCNY-NRG4* was identified in a clear cell carcinoma. The recurrent involvement of *CCNL2* in four fusions and of three other genes of the cyclin family in three additional transcripts hints that deregulation of cyclin genes is important in the pathogenesis of ovarian carcinomas in general but of endometrioid carcinomas particularly.

Malignant epithelial tumors (carcinomas) are the most common ovarian cancers and also the most lethal gynecological malignancies.[1] Based on histopathology and genetic profiling, ovarian carcinomas are divided into five main types: high-grade serous (HGSC) (representing 70% of the malignancies), endometrioid (EC) (10%), clear cell (10%), mucinous (3%), and low-grade serous carcinomas (LGSC) (<5%; the relative frequencies refer to data from Western countries); together they account for over 95% of ovarian malignant tumors.[2] Each of these histotypes differs in what is their precursor lesion(s), oncogenesis, response to chemotherapy, and prognosis.[3] HGSC harbor *TP53* and *BRCA* mutations, whereas their low-grade counterparts often carry *KRAS* and *BRAF* mutations. *KRAS* and *HER2* mutations are frequent in mucinous carcinomas, whereas in EC and clear cell carcinomas, *ARID1A* is frequently mutated.[3]

Several studies have focused on the identification of fusion genes in ovarian carcinomas. Although >700 samples have been analyzed so far, only a few recurrent transcripts were found, and always with a low rate of recurrence (0.5–5%). Two studies used the genomic data produced by the Cancer Genome Atlas project[4,5] to find that three fusion transcripts were recurrent in HGSC carcinomas: *CCDC6-ANK3* (found in 4 samples or 1% of the tumors), and *COL14A1-DEPTOR* and *KAT6B-ADK* (each found in 2 samples or 0.5%). Patch *et al.*[6] analyzed 114 samples from chemoresistant HGSC and found promoter swapping affecting the *SLC25A40-ABCB1* transcript in six samples (5%). Earp *et al.*[7] found *CRHR1-*

Key words: ovarian carcinoma, fusion transcript, NRG4, cyclin, CCNL2
Additional Supporting Information may be found in the online version of this article.
Conflict of Interest: The authors have no conflict of interest to disclose.
Grant sponsor: Norwegian Radium Hospital Foundation; **Grant sponsor:** John and Inger Fredriksen Foundation; **Grant sponsor:** Anders Jahre's Foundation through UNIFOR (University of Oslo)
DOI: 10.1002/ijc.31418

History: Received 2 Feb 2018; Accepted 20 Mar 2018; Online 6 Apr 2018
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What's new?

Chimeric genes formed by fusion of previously separate genes are associated with many malignant tumors, but rare in ovarian cancer. Here the authors performed transcriptome sequencing of different types of ovarian tumors and identify novel fusion genes, involving cyclin genes, the master regulators of the cell cycle. As most of these fusions were found in ovarian cancer of the endometroid type, which represent about 10% of all ovarian cancers, the data point to a novel role of cyclin deregulation in this specific cancer subtype.

KANSL1 to be the most frequent fusion transcript in their series (2.7% of all tumors). Our group recently reported the involvement of *DPP9* in two out of 18 samples of HGSC karyotypically characterized by rearrangements of chromosome 19.[8] Taken together, these results suggest that ovarian cancer is not characterized by highly recurrent fusion transcripts. It should be taken into account, however, that the majority of studies referred to above focused exclusively on HGSC meaning that the other histotypes have not yet been extensively analyzed. We therefore screened a series of 34 tumors representing the whole spectrum of ovarian malignant epithelial tumors to look for new recurrent fusion transcripts arising in non-HGSC tumors.

Material and Methods

Tumor material

The material consisted of fresh frozen samples from ovarian tumors surgically removed at The Norwegian Radium Hospital between 1999 and 2010. Samples from 34 ovarian carcinomas (including borderline tumors) were sequenced (two borderline, two low-grade serous, three mucinous, four clear cell, nine EC, and 14 HGSC). A second cohort of 113 samples was subsequently used to validate the results and test how frequent were the novel fusion transcripts that had been detected. The latter series consisted of 10 fibromas, 10 thecofibromas, 10 borderline epithelial tumors and 83 carcinomas of which 35 were HGSC, 16 mucinous, 18 EC, 10 clear cell and 4 low-grade serous. The study was approved by the regional ethics committee (Regional komité for medisinsk forskningsetikk Sør-Øst, Norge, https://helseforskning.etikkom.no/) and written informed consent was obtained from the patients.

RNA extraction

Total RNA was extracted using miRNeasy Kit (Qiagen, Hilden, Germany) and QIAcube (Qiagen). The concentration and purity of the RNA was measured with the Nanovue Spectrophotometer (GE Healthcare, Pittsburgh, PA). The RNA quality of the 34 samples sequenced was checked with Experion Automated Electrophoresis System using the RNA StdSens analysis kit (Bio-Rad Laboratories, Oslo, Norway).

High-throughput paired-end RNA-sequencing and bioinformatics analyses

Three micrograms of total RNA were sent for high-throughput paired-end RNA-sequencing at the Norwegian Sequencing Center, Ullevål University Hospital (https://www.sequencing.uio.no/) as described previously.[9] The software used for detection of fusion transcripts included Fusioncatcher v0.99.4e (https://github.com/ndaniel/fusioncatcher),[10] Chimerascan v0.4.5 (https://github.com/genome/chimerascan-vrl),[11] FusionMap 31.03.15 (https://omictools.com/fusionmap-tool),[12] and TopHat 2.0.9 (https://ccb.jhu.edu/software/tophat/index.shtml).[13] The candidate fusion transcripts obtained by bioinformatic analysis were checked using BLAST (http://blast.ncbi.nlm. nih.gov/Blast.cgi) and BLAT (https://genome.ucsc.edu/cgi-bin/hgBlat?command=start).

Reverse transcriptase-polymerase chain reaction (RT-PCR) and Sanger sequencing

One microgram of RNA was reverse transcribed using iScript Advance cDNA synthesis kit (Bio-Rad). To validate the fusion genes detected as part of the bioinformatic analyses, cDNA equivalent to 10 ng RNA was amplified using the TAKARA Premix Ex Taq (TaKaRa-Bio, Europe/SAS, Saint-Germain-en-Laye, France). The primers are listed in Supporting Information I. The PCR cycling program for all reactions was as follows: 30 sec at 94°C followed by 35 cycles of 7 sec at 98°C, 30 sec at 55°C, 60 sec at 72°C and a final extension for 2 min at 72°C. Expression of the housekeeping gene *ABL1* was monitored as cDNA quality control. We also tested our series of tumors for presence of a fusion gene *CDKN2D-WDFY2*. The primers and PCR conditions were as reported.[14] Three microliters of the PCR products were stained with GelRed (Biotium, Hayward, CA) and analyzed by electrophoresis through 1.0% agarose gel. The gel was scanned with G-Box (Syngene, Los Altos, CA) and the images were acquired using GeneSnap (Syngene). The remaining 22 µl of the amplified fragments were purified using the QIAquick PCR purification Kit (Qiagen). Direct sequencing was performed using the light run sequencing service of GATC Biotech (http://www.gatc-biotech.com/en/sanger-services/lightrun-sequencing.html) or the ABI3500 Genetic Analyzer (ThermoFiosher Scientific, Waltham, MA) using BigDye Terminator V1.1 cycle sequencing kit. The BLAST and BLAT programs were used for computer analysis of sequence data.

Results

RNA-sequencing gave informative results for all 34 samples. The subsequent bioinformatic analysis was also informative

Table 1. List of the candidate fusion transcripts

Sample	Diagnosis	Candidate fusion genes[1]		Location		Breakpoint (exons)		Fusion outcome
I	CCC	***CCNY***	***NRG4***	10p11.21	15q24.2	1	4	In frame
		MRPL21	***TADA2A***	11q13.3	17q12	6	7	In-frame
		MICALL1	*GGA1*	22q13.3	22q3	13	14	In-frame
II	E	***PCMTD1***	***CCNL2***	8q11.23	1p36.33	3	6	In-frame
III	E	***ANXA5***	***CCNA2***	4q27	4q27	3	3	In-frame
		PAK1	***GYLTL1B***	11q13.5	11p11.2	14	15	In-frame
		AP1M2	***HIP1***	19p13.2	7q11.23	10	31	Out-of-frame
		CTBP2	*DENND3*	10q26.13	8q24.3	1	6	In-frame
		NAIP	***OLCN***	5q13.2	5q13.2	2	5	In-frame
IV	E	***PDE4D***	***CCNB1***	5q12.1	5q13.2	1	2	In-frame
		MELK	***TMEM88***	9p13.2	9p13.3	10	11	In-frame
		RGS10	***ZMYM1***	10q26.11	1p34.3	3	3	In-frame
V	E	***PCMTD1***	***CCNL2***	8q11.23	1p36.33	3	6	In-frame
VI	E	***PCMTD1***	***CCNL2***	8q11.23	1p36.33	3	6	In-frame
VII	E	***PCMTD1***	***CCNL2***	8q11.23	1p36.33	3	6	In-frame
VIII	HGSC	*SCNN1A*	*CHD4*	12p13.31	12p13.31	8	26	In-frame
		TSPAN3	***NRG4***	15q24.3	15q24.2	6	4	In-frame
IX	HGSC	*TRIM68*	*NRG4*	11p15.4	15q24.2	7	4	In-frame
X	HGSC	*NCAPG2*	*RBPMS*	7q36.3	8p12	15	2	In-frame
		MBD2	*PERP*	18q21	6q23	2	2	Out-of-frame
		DHX30	***ABHD14B***	3p21	3p21.2	6	5	In-frame
		SNTB1	***ZNF250***	8q24.1	8q24.3	1	6	In-frame
		MAP3K10	*C19orf47*	19q13.2	19q13.2	5	7	In-frame
XI	HGSC	*FUT8*	*FNTB*	14q23.3	14q23.3	2	6	In-frame
		AP2B1	***ZNF512***	17q12	2p23.3	2	13	In-frame
		FARP2	***PPP1R7***	2q37.3	2q37.3	8	2	In-frame
		FAM160B1	*NHLRC2*	10q25.3	10q25.3	5	6	Out-of-frame
		CTIF	***MOB2***	18q21.1	11p15.5	7	4	In-frame
XII	HGSC	***MGEA5***	***KCNIP2***	10q24.32	10q24.32	7	4	In-frame
		FAM20C	*SUGCT*	7p22.3	7p14.1	3	14	Out-of-frame
XIII	HGSC	***ARHGAP35***	***UNC13A***	19q13.32	19p13.11	4	40	In-frame
		FGFR2	*FAM24B*	10q26.13	10q26.13	9	2	In-frame
		KDM5A	***NINJ2***	12p13.3	12p13.3	24	2	In-frame
		PDZD8	*ABLIM1*	10q26.11	10q25.3	2	2	In-frame
		HDAC7	*VDR*	12q13.11	12q13.11	16	3	Out-of-frame
XIV	HGSC	***VRK1***	***TDP1***	14q32.2	14q32.11	3	15	In-frame
		NSD1	***ZNF346***	5q35.3	3q35.2	3	6	In-frame
		NFIX	*RAD23A*	19p13.3	19p13.3	2	1	In-frame
		PRKD1	*CNIH1*	14q12	14q22.2	1	2	In-frame
		TMEM123	*MMP27*	11q22.2	11q22.2	2	7	In-frame
		KLC1	*ZFAT*	14q32.33	8q24.22	1	8	In-frame

[1]The fusion transcripts that were validated with RT-PCR and Sanger sequencing are written in bold.
Abbreviations: CCC: clear cell carcinoma; E: endometrioid; HGSC: high-grade serous carcinoma.

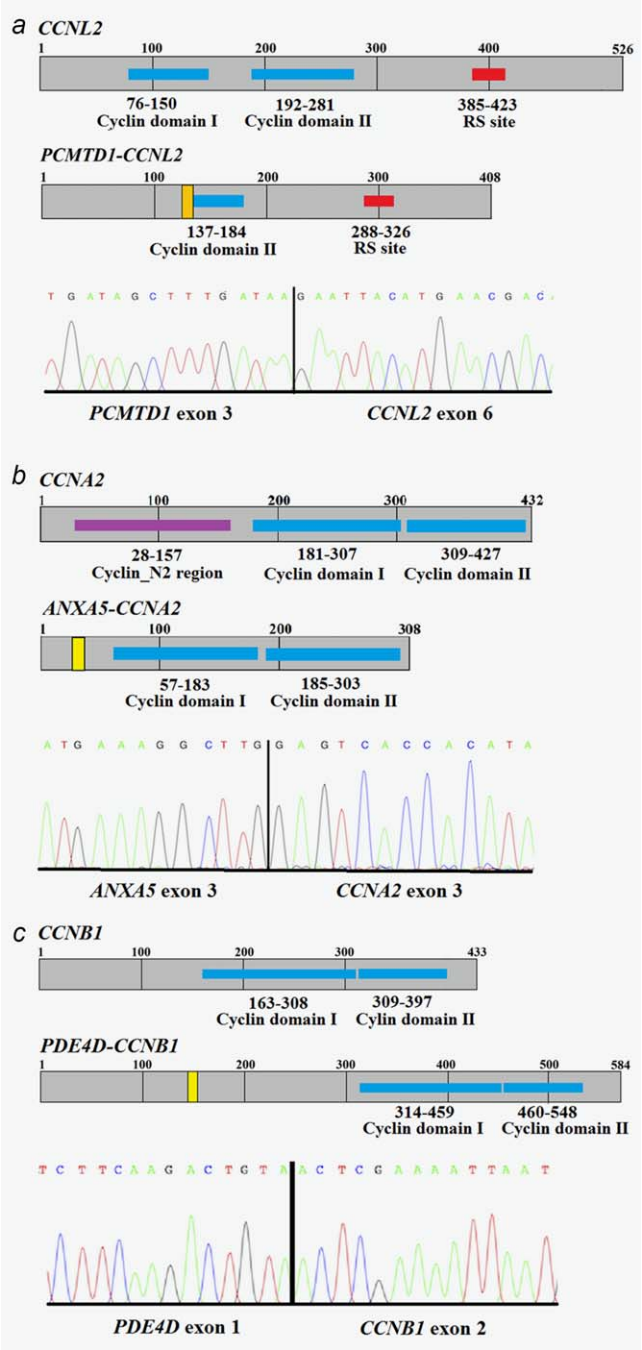

Figure 1. Schematic illustration of the putative chimeric proteins resulting from the detected fusions of cyclin genes. (*a*) Illustration of CCNL2 protein and the putative chimeric protein resulting from the *PCMTD1-CCNL2* fusion gene with a chromatogram showing the fusion junction identified by Sanger sequencing. (*b*) Wild-type CCNA2 and putative fusion protein translated from *ANXA5-CCNA2* with a chromatogram showing the fusion junction. (*c*) CCNB1 protein illustration and the putative chimeric protein encoded by the fusion gene *PDEA4D-CCNB1* with a chromatogram of the fusion junction. [Color figure can be viewed at wileyonlinelibrary.com]

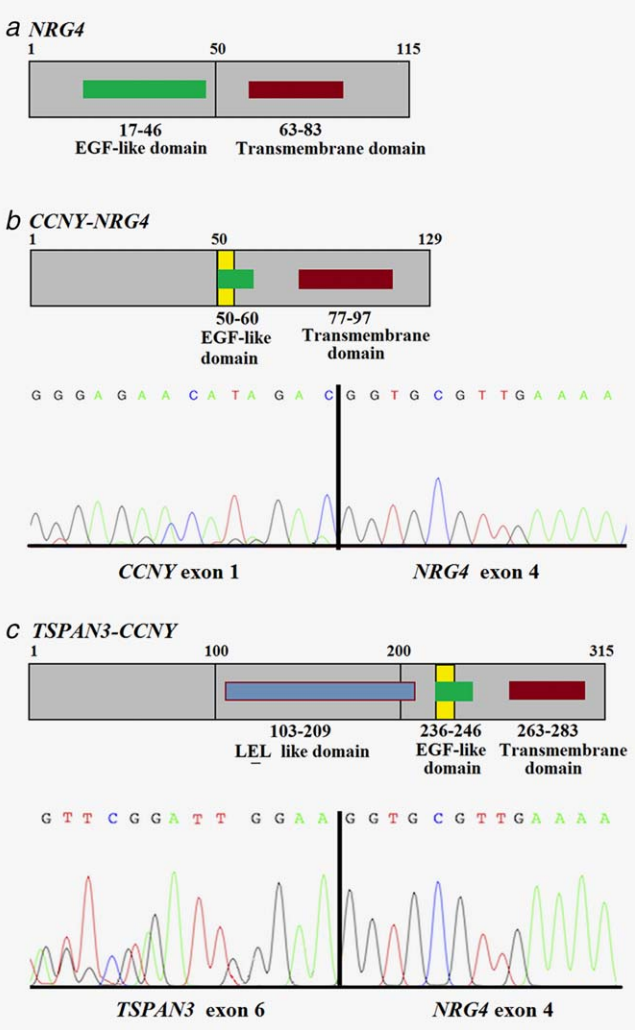

Figure 2. Schematic illustration of the putative chimeric *NRG4*-encoded proteins. (*a*) Illustration of the Neuregulin 4 (NRG4) protein. (*b*) Putative chimeric NRG4 resulting from the *CCNY-NRG4* fusion and chromatogram showing the fusion junction identified by Sanger sequencing. (*c*) Putative chimeric NRG4 resulting from the *TSPAN3-NRG4* fusion and chromatogram showing the fusion junction. [Color figure can be viewed at wileyonlinelibrary.com]

giving a mean of four fusion transcripts per tumor. The list of fusion candidates was shortened by checking every transcript with the BLAST and BLAT programs. All fusion sequences that did not involve the coding regions of both genes (3′UTR-coding DNA sequence (CDS), intronic–CDS and/or intronic–intronic), were discarded as were sequences identified as read-throughs. We focused on transcripts involving genes known to be relevant in cancer and transcripts that were identified by more than one program, the only exception being *PCMTDI-CCNL2* which was identified only by TopHat. Using these criteria, we came up with a list of 42 candidate fusion transcripts (Table 1) present in 11 samples out of 34 sequenced. More specifically, we found seven fusions in 14 HGSC, three in nine EC and one in the four clear cell carcinomas analyzed.

Twenty-two out of the 39 candidate fusion genes could be validated by PCR and Sanger (direct) sequencing (Table 1). The uniqueness of these fusion transcripts was checked against the Mitelman Database for Chromosome Aberrations

and Gene Fusions in Cancer (https://cgap.nci.nih.gov/Chromosomes/Mitelman). Only the fusion transcripts *KDMA5-NINJ2* and *NSD1-ZNF346* were previously identified, by Yoshihara *et al.*[5] in HGSC. All other fusion genes were novel.

Table 2. Overview of the expression status, at RNA and protein level, of the genes found involved in fusion events

Gene	RNA expression Illumina BodyMap 2.0[1]	Protein expression[2] (normal tissue)	Protein expression (cancer samples)[3]
PCMTD1	48	Low	Medium
CCNL2	59	Medium	Medium
ANXA5	156	Medium	Low
CCNA2	10	Not detected	Low
PDE4D	21	Medium	Medium
CCNB1	15	Not detected	Medium/low
CCNY	70	Low	Medium/low
TSPAN3	143	Not detected	Medium/low
NRG4	13	Not detected	Low

[1]Ovarian tissue RNA expression from Illumina Human BodyMap 2.0 dataset in reads per kilobase million (RPKM) assessed on 70 samples of ovarian normal tissue. Source: GeneCards (http://www.genecards.org).
[2]Protein expression of normal ovarian tissue assessed by immunohistochemistry. Source: the Human Protein Atlas (http://www.proteinatlas.org).
[3]Protein expression in 12 samples of ovarian cancer assessed by immunohistochemistry. Source: the Human Protein Atlas.

We found recurrent involvement of genes belonging to the cyclin family in endometrioid carcinomas in the form of fusion transcripts *PCMTDI-CCNL2, ANXA5-CCNA2* and *PDE4D-CCNB1* (Figure 1). Furthermore, *CCNY-NRG4,* another fusion involving a cyclin gene, was found in a tumor showing mixed endometrioid/clear cell histotype in its primary location (uterus) but only a clear cell pattern in the ovarian recurrence (see below). In addition, we found two transcripts involving the neuregulin 4 (*NRG4*) gene, the already mentioned *CCNY-NRG4* and *TSPAN3-NRG4*; the latter was found in an HGSC. In both cases, the fusion involved exon 4 of *NRG4* (Figure 2). The bioinformatic analyses identified an additional fusion transcript involving exon 4 of *NRG4* and exon 7 of the Tripartite Motif Containing 68 gene (*TPRMS68*) in another sample of HGSC; however, we could not confirm the presence of this transcript by means of PCR and sequencing analysis.

As all these fusion transcripts were non-recurrent in the original series of 34 tumors subjected to NGS, we also tested a larger cohort of 113 ovarian tumors for their possible presence. *PCMTD1-CCNL2* was found in three additional cases of EC (thus it was found present in four out of 18 carcinomas of the endometrioid histotype in total; 22%). The *PCMTD1-CCNL2* in-frame fusion juxtaposes exon 3 of the Protein-L-Isoaspartate (D-Aspartate) O-Methyltransferase Domain Containing 1 gene (*PCMTD1*; accession number: NM_052937.3) from 8q11.23 with exon 6 of the Cyclin L2

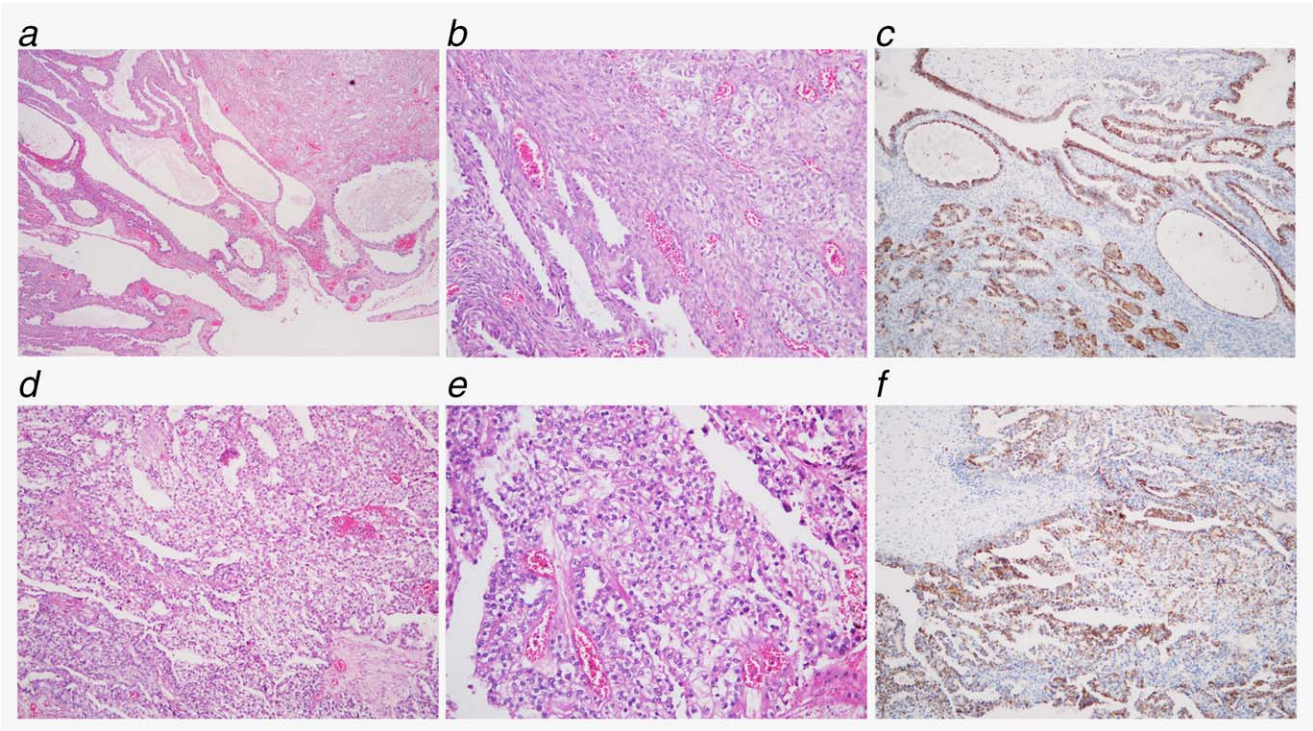

Figure 3. Histological appearance of the tumor of Case I. Hematoxylin–eosin staining at (*a*) 50× and (*b*) 200× magnification and (*c*) Napsin A immunostaining of the primary uterine carcinoma with a mixed clear cell-endometrioid morphology. Hematoxylin eosin staining at (*d*) 50× and (*e*) 200× magnification and (*f*) Napsin A immunostaining of the secondary clear cell carcinoma in the ovary. [Color figure can be viewed at wileyonlinelibrary.com]

Table 3. Overview of genes found involved in chimeric transcripts in this series and in previous studies

Gene	Type of Cancer
ABDH14B	Breast[5]
AP2B1	Lung, breast[5]
ARHGAP35	Breast[5]
CCNB1	Osteosarcoma,[33] breast[5]
CCNY	Prostate,[34] breast,[5] kidney, bladder, thyroid[35]
CTIF	Lung, breast[5]
DHX30	Breast[5]
FARP2	Prostate,[34] lung[31]
KDM5A	Acute myeloid leukemia,[36] breast, lung, kidney[5]
MELK	Breast[5]
MGEA5	Soft tissue tumors[37,38]
NINJ2	Lung, brain, ovary, breast[5]
NRG4	Breast[5]
NSD1	Acute myeloid leukemia,[39] ovary, lung, breast[5]
PAK1	Ovary,[5] breast[40]
PCMTD1	Acute lymphocytic leukemia,[41] ovary, prostate, lung, breast[5]
RBPMS	Lung, breast,[5] kidney,[40] thyroid[35]
SNTB1	Ovary, breast[5]
TSPAN3	Breast[5]
UNC13A	Brain, lung[5]
VRK1	Lung, oral cavity[5]
ZNF512	Ovary, breast[5]

gene (*CCNL2*; NR_135154.1) from 1p36.33 (Figure 1). The putative chimeric transcript is 3209 bp long and consists of a sequence of 762 bp from *PCMTD1* fused with a sequence of 2447 bp from *CCNL2*. It codes for a chimeric protein of 409 aa containing the first 136 aa (1–136 out of 357) of PCMTD1 (NP_443169) and 273 aa (253–526 out of 526) of Cyclin L2 (NP_112199.2).

No other fusion was found to be recurrent. We also tested the cohort for presence of the *CDKN2D-WDFY2* transcript that was previously reported with a recurrence rate of 20% in HGSC,[14] finding no such fusion.

Discussion

Studies over the past decades have uncovered the oncogenic role of fusion genes in hematological malignancies and mesenchymal tumors, and have highlighted the diagnostic and therapeutic advantages provided by the detection of these chimeric transcripts and their tumor-specific expression.[15] A similar search for fusion transcripts in ovarian cancer has shown that they are not common. Though the fusions *ESSRA-C11orf20*,[16] *CDKN2D-WDFY2*[14] and *BCAM-AKT2*[17] were initially described as recurrent in HGSC at rates of 15%, 20% and 7%, respectively, the findings have not been validated by other groups or in different series.[18] More than 700 samples were screened in other studies and fusion transcripts were found at frequencies ranging from 0.5% (*KAT6B-ADK*) to 2.7% (*CRHR1-KANSL1*).[4–7] It is worthy of note in the context that most studies focused on HGSC, whereas the other four types of ovarian carcinoma were less often investigated; only 63 endometrioid carcinomas, 36 clear cell carcinomas, and six mucinous carcinomas had been analyzed for fusion genes prior to this study.[7,16] In a recent study, Earp *et al.*[7] identified *UBAP1-TGMT* in clear cell carcinomas exclusively, finding it in two out of 20 tumors of this histotype.

We identified *PCMTD1-CCNL2* as a novel and recurrent fusion in endometrioid carcinomas, finding the transcript in four out of 18 (22%) EC. The *CCNL2* gene encodes three cyclin L2 isoforms.[19] The main isoform (Cyclin L2α) contains two cyclin domains, spanning amino acids 76–150 and 192–281, and a C-terminal RS site (arginine–serine dipeptide) (385–423) that plays a role in protein–protein interactions with the SR family of splicing factors.[20] The three splicing variants L2βA1/2/3 have exon 6 as the last coding exon and code for the 226 aa Cyclin L2βA isoform. The variant L2βB terminates in exon 7 and codes for the 236 aa Cyclin L2βB. Cyclin L2, which is different from most other cyclins, is expressed during the entire cell cycle and was detected in many tissues, ovary included (Table 2). Cyclin L2 participates together with Cyclin L1 and CDK11 in pre-mRNA splicing processes.[19,20] Lack of a functional Cyclin L2 may impair normal splicing mechanisms as all three Cyclin L2 isoforms have been shown to be fundamental components of the splicing complex.[19]

The role of Cyclin L2 in cancer has not been investigated extensively; however, Li *et al.*[21] showed that it acts as a tumor suppressor protein in gastric cancer enhancing both apoptosis and chemosensitivity. Yang *et al.*[20] showed a similar tumor suppressor activity of Cyclin L2 in hepatocarcinoma and that both cyclin domains were fundamental for the protein's proper functioning.[20] The fusion *PCMTD1-CCNL2* leads to a chimeric Cyclin L2α lacking the first cyclin domain (76–150) and containing only 27 aa of the second cyclin domain (Figure 1). Due to the fusion, the chimeric protein is no longer able to bind CDK11 and suppress tumor growth. The fusion leads to loss of the other two isoforms since the splicing variants coding for isoforms L2βA/B are lost.

Besides the aforementioned fusion, we found also another two fusion transcripts, *ANXA5-CCNA2* and *PDE4D-CCNB1*, involving other cyclin genes in two different samples of EC. The in-frame fusion *ANXA5-CCNA2* juxtaposes exon 3 of the Annexin A5 gene (*ANXA5*; NM_001154.3, from 4q27) with exon 3 of the gene coding for Cyclin A2 (*CCNA2*; NM_001237.3, mapping in the same genomic region only 160 kb more distal). The fusion results in a 2250 bp (259 bp from *ANXA5* and 1991 bp from *CCNA2*) transcript which codes for a functional chimeric cyclin composed of 31 aa

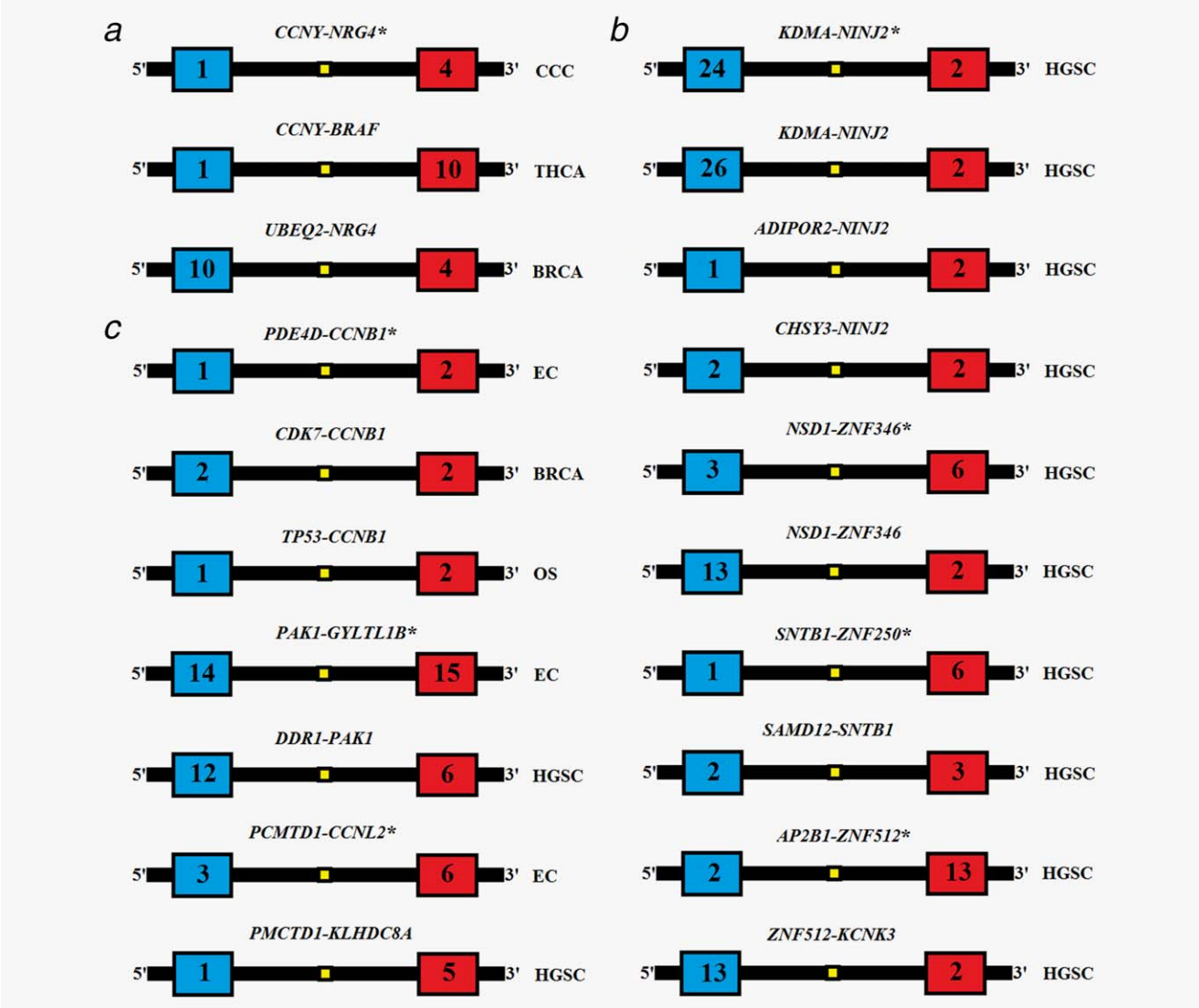

Figure 4. Schematic illustration of the fusion junctions of the recurrent fusion genes described in our series and in the literature. Fusion junctions of the chimeric transcripts found in (*a*) CCC, (*b*) HGSC, and (*c*) EC of the present series and fusion transcripts from the literature involving one of the gene with different partners or the same genes with different breakpoints.[5,33,35] The asterisk (*) indicates the fusion transcripts detected in the present series. The yellow square indicates the fusion junction. The numbers in the blue and red boxes indicate the exons brought together by the fusion. Thyroid cancer (THCA), breast cancer (BRCA), and osteosarcoma (OS). [Color figure can be viewed at wileyonlinelibrary.com]

(1–31 out of 320) from ANXA5 (AAH01429.1) and 274 aa (158–432 out of 432) from Cyclin A2 (AAI04784.1) (Figure 1). The functional cyclin domains of Cyclin A2 are located in regions 181–307 and 309–427 (Figure 1) and are therefore conserved in the chimeric cyclin encoded by *ANXA5-CCNA2*. The fusion gene *PDE4D-CCNB1* brings together exon 1 of the Phosphodiesterase 4 D gene (*PDE4D*; NM_001197223.1) from 5q11.2 and exon 2 of a gene coding for Cyclin B1 (*CCNB1*, NM_031966.3) from 5q13.2. The fusion results in a chimeric transcript of 1886 bp (627 bp from *PDE4D* and 1886 bp from *CCNB1*) which codes for a putative protein of 584 aa containing 151 aa (1–151 out of 809) from phosphodiesterase 4 D (NP_001098101) and the whole Cyclin B1 (433aa) (AAP88038) (Figure 1). The consequences of these two fusions should be similar despite the fact that they affect different genes.

Cyclin genes act in concert.[22] The expression levels of Cyclin A2 are tightly synchronized with cell cycle progression. *CCNA2* transcription begins in late G1, peaks and plateaus in mid-S, and then declines in G2.[23] The transcription is mostly regulated by the transcription factor E2F that derepresses the promoter.[24] Cyclin B1 appears in S phase and accumulates in G_2 and mitosis before disappearing at transition from metaphase to anaphase. Synthesis of Cyclin B1 during the cell cycle is mainly regulated at the transcriptional level by p53[25] and is enhanced by the P300 coactivator,[26] USF1,[27] and Myc.[28] The fusions *ANXA5-CCNA2* and *PDE4D-CCNB1* bring the cyclins under the control of the

promoter of their 5′ partners that are normally expressed in ovarian cells (Table 2). This promoter swapping overcomes the normal regulation of the cyclin genes resulting in deregulation, that is, overexpression/permanent expression of chimeric Cyclins A2 and B1. This may profoundly affect cell cycle regulation since these two cyclins cooperate in both early and late mitosis.[29]

The *CCNY-NRG4* fusion transcript was found in a clear cell ovarian carcinoma. In this transcript, exon 4 of *NRG4* is fused with exon 1 of *CCNY*, that is, another cyclin gene. The fusion causes the complete loss, at the genomic level, of the entire cyclin gene (*CCNY*; NM_181698.3), replacing it by *NRG4* (exons 4–6; NM_138573.3). Despite the fact that the metastatic tumor sample (the one examined) showed clear cell histology, it is interesting to note that its primary in the uterus had mixed morphology with clear cell and endometrioid morphology (Figure 3). Presence of the latter phenotype again seems consistent with involvement of cyclin genes in fusion transcripts in tumors showing endometrioid features.

The *NRG4* gene was found rearranged also with another partner, Tetraspanin-3 (*TSPAN3*). The transcript consisted of exon 6 from *TSPAN3* fused with exon 4 from *NRG4* (similar to *CCNY-NRG4*). The gene *NRG4* is located on chromosomal band 15q24 and codes for Nereugulin 4 (CAL35829.1), a ligand of the EGF receptor family. Whereas the Neuregulin 4 gene is not expressed at high levels in the normal ovary (13 RPKM), *CCNY* (70 RPKM), and *TSPAN3* (143 RPKM) are consistently transcribed (Table 2). Fusion between these genes results in an increased level of the chimeric NRG4. The *NRG4* gene codes for a 115 aa protein which contains two main functional domains: an extracellular EGF-like domain (17–46 aa) and a transmembrane domain (63–83 aa) (Figure 2). The EGF-like domain is fundamental in the activation of EGF family receptors HER4 and HER3.[30] In the *CCNY-NRG4* and *TSPAN3-NRG4* fusion transcripts, the EGF-like domain of *NRG4* is partially lost as only 10 out of 29 aa are conserved in the chimeric protein while the transmembrane domain is conserved (Figure 2). These findings suggest that *NRG4* is recurrently found involved in fusion transcripts as it was rearranged in 8.8% of the samples analyzed by RNA-sequencing (3 out of 34).

Even though the results obtained are limited and our preliminary conclusion should be borne out in larger studies, the presented evidence clearly hints that cyclin fusion transcripts play a role in the pathogenesis of a subset of EC.

All other fusion transcripts validated using PCR were nonrecurrent in our series. However, taking into account information from the Mitelman database (http://cgap.nci.nih.gov/Chromosomes/Mitelman) we found that some of the fusion genes identified in this study have indeed been previously reported, both in ovarian cancer and other tumors (Table 3 and Figure 4), albeit in some cases with a different partner. The fusions *KDMA5-NINJ2* and *NSD1-ZNF346* were previously identified in HGSC as was seen in our case (Figure 4). The genes *NINJ2*, *PAK1, PCMTD1, SNTB1*, and *ZNF512* were also found fused with different partners in HGSC (Figure 4).[5] Taking all these results together, we see that all the mentioned genes were recurrently rearranged in ovarian cancer, admittedly at very low frequencies. Furthermore, 21 of the genes we found involved in fusions were previously reported in 35 different fusion transcripts in studies of breast cancer and 14 fusion transcripts were identified in lung cancer[5,31,32] (Table 3), hinting that they may be generally relevant in carcinogenesis of different types.

As we did not find any sample carrying the *CDKN2D-WDFY2* fusion and no evidence for the presence of *ESSRA-C11orf20* and/or *BCAM-AKT2* in our series, we conclude that the frequency of these fusions must be much lower than was initially reported.[14,16,17] Indeed, it seems evident that the occurrence of pathogenetically essential fusion events in ovarian carcinomas is well below what is the case in hematological malignancies and/or mesenchymal tumors.[42] The fusion genes found in this and other studies may also be a reflection of massive pathogenetic heterogeneity in ovarian cancer, thus identifying tumor subsets within, but on other occasions transcending, the accepted phenotypic subgroups of ovarian cancer. One may hope that further elucidation of this tumorigenic variability will contribute to a more meaningful classification of these malignancies and eventually to the finding of medicines directed at the molecular genetic changes that are central to the disease process.

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