CAMK2N1/RUNX3 methylation is an independent prognostic biomarker for progression-free and overall survival of platinum-sensitive epithelial ovarian cancer patients

Karolin Heinze¹, Matthias Rengsberger¹, Mieczyslaw Gajda², Lars Jansen¹, Linea Osmers¹, Leticia Oliveira-Ferrer³, Barbara Schmalfeldt³, Matthias Dürst¹, Norman Häfner¹* and Ingo B. Runnebaum¹*

Abstract

Background: To date, no predictive or prognostic molecular biomarkers except BRCA mutations are clinically established for epithelial ovarian cancer (EOC) despite being the deadliest gynecological malignancy. Aim of this biomarker study was the analysis of DNA methylation biomarkers for their prognostic value independent from clinical variables in a heterogeneous cohort of 203 EOC patients from two university medical centers.

Results: The marker combination CAMK2N1/RUNX3 exhibited a significant prognostic value for progression-free (PFS) and overall survival (OS) of sporadic platinum-sensitive EOC (n = 188) both in univariate Kaplan–Meier (LogRank p < 0.05) and multivariate Cox regression analysis (p < 0.05; hazard ratio HR = 1.587). KRT86 methylation showed a prognostic value only in univariate analysis because of an association with FIGO staging (Fisher’s exact test p < 0.01). Thus, it may represent a marker for EOC staging. Dichotomous prognostic values were observed for KATNAL2 methylation depending on BRCA aberrations. KATNAL2 methylation exhibited a negative prognostic value for PFS in sporadic EOC patients without BRCA1 methylation (HR 1.591, p = 0.012) but positive prognostic value in sporadic EOC with BRCA1 methylation (HR 0.332, p = 0.04) or BRCA-mutated EOC (HR 0.620, n.s.).

Conclusion: The retrospective analysis of 188 sporadic platinum-sensitive EOC proved an independent prognostic value of the methylation marker combination CAMK2N1/RUNX3 for PFS and OS. If validated prospectively this combination may identify EOC patients with worse prognosis after standard therapy potentially benefiting from intensive follow-up, maintenance therapies or inclusion in therapeutic studies. The dichotomous prognostic value of KATNAL2 should be validated in larger sample sets of EOC.

Keywords: Epithelial ovarian cancer, Prognostic marker, DNA methylation, RUNX3, CAMK2N1 (CaMKIINα)

Background

Despite all medical efforts and advances, epithelial ovarian cancer (EOC) remains the most lethal gynecological malignancy. In Germany, this disease accounts for 3.2% of all newly diagnosed cancer cases and 5.3% of all cancer-related death of women [1]. The lifetime risk of developing this cancer entity lies by 1–2%. There are
several factors increasing or decreasing the lifetime risk. Demographic, environmental and hormonal facts possibly add to the risk. Additionally, various familiar factors influence the chances of developing EOC. Family cancer syndromes predispose patients for the development of this malignancy. A number of syndromes are associated with ovarian cancer, the hereditary breast and ovarian cancer (HBOC) syndrome being the most prominent one [2]. Within this syndrome, the genes BRCA1 and BRCA2 are frequently mutated [3, 4]. Germline mutations account for 15–25% of all ovarian carcinoma [5, 6] while somatic mutations of the BRCA genes are additionally detected in 5–6% of EOC [7, 8]. The five-year survival rate of EOC lies between 35 and 45% [1, 9–11]. These low survival numbers can be explained by the lack of early detection methods resulting in the frequent diagnosis of progressed tumor stages (≥ FIGO III). Standard therapy comprises surgery to reduce the tumor burden combined with platin-taxane-based chemotherapy. A successful surgical intervention is crucial for improved survival. A macroscopic completely resected tumor is most preferable and improves the odds of survival and therapy success [12, 13]. Whereas approximately 20% of patients exhibit intrinsic resistant EOC most tumors of initially sensitive patients develop platin resistance and relapse within a median of 18 month [14, 15]. Neither prognostic markers for EOC nor predictive markers for standard chemotherapy are clinically established. Patients with BRCA mutations tend to respond better to platin and show improved progression-free survival but this does not necessarily translate into improved long-term overall survival [16, 17]. Additionally, BRCA mutations or homologous repair defects (HRD) can identify patients benefitting from PARPi treatment [18–21]. However, even patients with HRD-proficient tumors can be successfully treated with PARPi [19, 22] pointing to the potential requirement to establish better predictive marker. Moreover, no predictive marker is defined for anti-angiogenesis treatment with Bevacizumab. Thus, in the absence of predictive biomarker for any adjuvant treatment, prognostic biomarker may help to identify patients with worse outcome benefitting from maintenance treatment with these new treatment options. Both clinical parameters and different molecules or cell populations are targets of intense research to identify and validate prognostic marker. Clinical parameters can be analyzed posttreatment only (resection status, tumor stage, chemotherapeutic response) but are well established [23]. Biomarker may be analyzed pre-therapeutically (blood-based liquid biopsy) or at least before start of adjuvant chemotherapy (tissue-based biomarker) but are far from being implemented in clinical routine. Biomarker classes for liquid biopsies can be circulating nucleic acids, immune parameters, serum proteins or extracellular vesicle carrying miRNA and other molecules [24–28]. Tissue-based marker may consist of aberrant DNA, coding- or non-coding RNA expression or the analysis of intratumoral immune cell populations [7, 29–34]. One DNA aberration which can be used as molecular biomarker is CpG hypermethylation belonging to the field of epigenetics.

Epigenetic changes influence gene expression in a reversible manner without changing the DNA sequence. Histone modification, DNA methylation and, micro-RNA associated silencing are modes of epigenetic alteration in both normal developmental processes and disease, e.g., cancer development [35, 36]. DNA methylation is a stable epigenetic modification catalyzed by DNA-Methyltransferases (DNMT’s) and recognized by methyl-CpG binding proteins (MBDs) resulting in changed chromatin states. Since 5-methyl-cytosines and the DNA itself are highly stable, efforts to use these as biomarker are the focus of current medical research [37–39]. Epigenetic changes are seen in various tumor entities, ovarian cancer being one of them [40]. Currently, aberrant DNA methylation in EOC is detected using different approaches such as target-specific methylation-specific PCR (MSP) [41] or whole genome approaches [42–44]. A large number of genes are affected by DNA methylation in EOC [40]. For instance, tumor suppressor genes such as BRCA1, RASSF1A [45] or MLH1 [41, 46]; cell adhesion genes as ICAM-1 [47] and CDH1 [48] and DNA repair genes PALB2 [49] are affected by DNA methylation. BRCA1 promoter methylation is reported in 10–15% of sporadic OvCa [7, 50–53]. It appears that a reversal of this phenotype can take place over time leading to changed methylation level in relapsed disease [54]. MLH1 methylation is potentially linked to chemotherapy response or resistance development [46]. However, lack of candidate biomarker validation and low sensitivity or specificity prevents the clinical use of methylation-based biomarker [55].

In previous studies, we screened twelve primary high-grade serous ovarian carcinomas (HGSOC) using genome wide CpG microarrays for differentially methylated genes to identify potential prognostic biomarkers. 37 hypo-/hypermethylated regions were selected to verify promising candidates in a comparative cohort analysis of 36 samples (PFS< 3 years vs. >3 years). The methylation marker genes ATL1, CAMK2N1, KATNAL2, KRT86 and RUNX3 were detected as most promising due to good discriminatory power and the combination CAMK2N1 and RUNX3 could identify EOC patients with significantly shortened PFS in univariate analysis [44]. However, the small number of patients and missing multivariate analyzes for the prognostic value related to PFS and OS were limitations of this study. Nevertheless,
subsequent functional analyses identified tumor suppressive functions for \textit{CAMK2N1} and a dichotomous role for \textit{RUNX3} transcript variants regulated by DNA methylation \cite{56}. Robust validation and proof of an independent prognostic value are crucial before biomarkers are tested prospectively in a clinical setting. Therefore, this study aimed to validate the previously identified and most promising epigenetic candidate genes—\textit{ATL1}, \textit{CAMK2N1}, \textit{KATNAL2}, \textit{KRT86} and \textit{RUNX3}—using a larger patient cohort from two different University hospitals enabling multivariate analyses. To provide further information for patients with a hereditary form of ovarian cancer, a small number of such patients were also included and the \textit{BRCA1} methylation status was additionally evaluated in all samples.

\textbf{Results}

\textbf{Methylation analysis of biomarker candidate genes}

Patients from two university hospital centers were included in this retrospective analysis. Inclusion criteria were histologically confirmed primary epithelial ovarian cancer, available fresh frozen tissue and at least 36 months follow-up data. After screening of the accessible information and material, samples were excluded due to underrepresentation of tumor cells (<10%) and tissue derived from relapse events. A total of 203 tumor samples existed for the validation of biomarker candidate genes. Samples from patients with known or identified \textit{BRCA1/2} mutations (7.5%; \textit{n} = 15) were evaluated in a separate analysis (see below) to exclude any bias caused by the known prognostic value of \textit{BRCA} mutations \cite{16}. Basic clinical data from the analyzed patients are shown in Table 1.

The cohort from Jena University Hospital (\textit{n} = 101) consists of 65 new patients but also includes 36 samples from our previous study \cite{44}. To enable a correct validation, all samples used for initial marker identification were excluded. To validate the robustness of employed MSP assays and to exclude major bias by tumor heterogeneity, several experiments were done. First of all, a subset of samples analyzed in Häfner et al. \cite{44} was re-tested. Since several years and different other analyses were performed using the very same tumor block, a deeper section of the tumor was analyzed. Secondly, from some patients, multiple biopsy tissues were available. Both analyses revealed a high consistency of the methylation pattern throughout the samples (95% concordance, Cohen's Kappa = 0.881) proving the validity and robustness of methylation marker detection (Additional file 1: Fig. S1).

Altogether the cohort reflects the typical EOC patient and mainly comprises of late stage HGSOC patients who were treated by macroscopically complete resection and

\begin{table}[h]
\centering
\caption{Clinical characteristics of analyzed EOC patients}
\begin{tabular}{lll|ll}
\hline
Criteria & Sporadic EOC & & Mutated EOC & \\
 & \textit{n} & \% & \textit{n} & \% \\
\hline
\textit{FIGO} & & & & \\
I & 13 & 6.9 & 3 & 20.0 \\
II & 6 & 3.2 & 0 & 0.0 \\
III/IV & 165 & 87.8 & 12 & 80.0 \\
Missing & 4 & 2.1 & 0 & 0.0 \\
\textbf{Grading} & & & & \\
G1 & 6 & 3.2 & 3 & 20.0 \\
G2 & 29 & 15.4 & 2 & 13.3 \\
G3 & 147 & 78.2 & 10 & 66.7 \\
Missing & 6 & 3.2 & 0 & 0.0 \\
\textit{pT} & & & & \\
T1a & 6 & 3.2 & 1 & 6.7 \\
T1b & 1 & 0.5 & 0 & 0.0 \\
T1c & 7 & 3.7 & 2 & 13.3 \\
T2a & 8 & 4.3 & 0 & 0.0 \\
T2c & 7 & 3.7 & 0 & 0.0 \\
T3a & 8 & 4.3 & 0 & 0.0 \\
T3b & 20 & 10.6 & 2 & 13.3 \\
T3c & 129 & 68.6 & 10 & 66.7 \\
Missing & 2 & 1.1 & 0 & 0.0 \\
\textit{pN} & & & & \\
N0 & 60 & 31.9 & 5 & 33.3 \\
N1 & 114 & 60.6 & 7 & 46.7 \\
Nx & 14 & 7.4 & 3 & 20.0 \\
\textit{pM} & & & & \\
M0 & 137 & 72.9 & 12 & 80.0 \\
M1 & 35 & 18.6 & 3 & 20.0 \\
Mx & 16 & 8.5 & 0 & 0.0 \\
\textbf{Histology} & & & & \\
Serous & 156 & 83.0 & 13 & 86.7 \\
Endometrioid & 15 & 8.0 & 0 & 0.0 \\
Mucinous & 3 & 1.6 & 2 & 13.3 \\
Other & 14 & 7.4 & 0 & 0.0 \\
\textbf{Resection} & & & & \\
R0 & 141 & 75.0 & 12 & 80.0 \\
R1/R2 & 42 & 22.3 & 3 & 20.0 \\
Missing & 5 & 2.7 & 0 & 0.0 \\
\textbf{Chemotherapy} & & & & \\
Carbo/Pac & 150 & 79.8 & 13 & 86.7 \\
Carbo & 8 & 4.3 & 0 & 0.0 \\
Carbo/Pac+x & 11 & 5.9 & 0 & 0.0 \\
Carbo+x & 8 & 4.3 & 1 & 6.7 \\
Other & 3 & 1.6 & 0 & 0.0 \\
None & 7 & 3.7 & 1 & 6.7 \\
Missing & 1 & 0.5 & 0 & 0.0 \\
\textbf{Bevacizumab} & & & & \\
Yes & 29 & 15.4 & 5 & 33.3 \\
No & 157 & 83.5 & 10 & 66.7 \\
Missing & 2 & 1.1 & 0 & 0.0 \\
\textbf{Complete} & 188 & 100.0 & 15 & 100.0 \\
\hline
\end{tabular}
\end{table}
platin-taxane-based chemotherapy (Table 1). However, the cohort is more diverse than the establishing cohort in regard to tumor stage, histology, surgical resection status and adjuvant chemotherapy. Specifically, a subset of patients received maintenance treatment with Bevacizumab, currently standard of care for advanced stage EOC patients in Germany [57]. Evaluating the clinical parameters of the 188 included sporadic EOC patients, we confirmed the prognostic value of the FIGO staging (FIGO I+II vs. FIGO III+IV) and surgery outcome (tumor residual 0 mm vs. <10 mm/ >10 mm) for overall survival (OS) and progression-free survival (PFS) (Table 2; Additional file 1: Fig. S2; LogRank \( p < 0.05 \)). High-grade serous EOC tend to show a shorter PFS than other EOC (\( p = 0.08 \)). The differentiation stage of the tumors (G1/2 vs. G3) did not show a prognostic value for PFS or OS.

Table 2 summarizes, in addition to the clinical parameters, the median and 95% confidence interval of PFS and OS as determined by univariate Kaplan–Meier analyzes for patients stratified by methylation biomarkers or combinations thereof. Combinations reflect the methylation status of at least one included marker (logical \(<\text{OR}>\) combination). Methylation of CAMK2N1, KRT86 and the combinations CAMK2N1/RUNX3 or CAMK2N1/RUNX3/KRT86 stratified patients into groups with significantly different PFS (Fig. 1, Table 2).

This confirmed our earlier results for these markers, whereas the prognostic value of the singular RUNX3 methylation could not be validated (Fig. 1, Table 2). Albeit KRT86 methylation showed the most significant discriminatory power in univariate analysis, it exhibited no independent prognostic value (\( p = 0.055 \), Table 3).

This is mainly related to the exclusive methylation in late stage EOC and the resulting significant association of KRT86 methylation with FIGO staging (Fisher’s exact test, \( p < 0.01 \)). The present large cohort with overall survival data enabled the additional determination of

### Table 2 Results of univariate LogRank analyses of clinical and molecular variables

| Criteria | Status | n [%] | PFS [month] Median 95% CI LogRank | OS [month] Median 95% CI LogRank |
|----------|--------|------|-----------------------------------|----------------------------------|
| FIGO     | I/II   | 19   | 10.4 Not reached                  | 145                              |
|          | III/IV | 163  | 89.6 29 25.5–32.5 \( p < 0.001 \) | 62                               |
| Resection| R0     | 139  | 76.8 38 28.3–47.8 \( p < 0.001 \) | 78                               |
|          | R1/2   | 42   | 23.2 20 13.9–26.1 \( p < 0.001 \) | 49                               |
| Histology| Serous | 154  | 82.8 30 26.6–33.4 \( p < 0.001 \) | 78                               |
|          | Non-serous | 32   | 17.2 58 0–121.1 \( p < 0.001 \)   | 64                               |
| Methylation | No marker | 77   | 41.4 31 24.7–34.7 \( p < 0.001 \) | 78                               |
|          | \( \geq \) 1 marker | 109  | 58.6 31 27.4–34.7 \( p < 0.001 \) | 62                               |
|          | CAMK2N1 | U | 170 91.4 31 25.2–36.8 \( p < 0.001 \) | 74                               |
|          | M     | 16   | 8.6 16 9.3–22.8 \( p < 0.001 \)   | 47                               |
|          | ATL1  | U   | 159 85.5 31 25.2–36.8 \( p < 0.001 \) | 64                               |
|          | M     | 27   | 14.5 27 20.2–33.8 \( p < 0.001 \)   | 65                               |
|          | KATNAL2 | U   | 111 59.7 33 23.3–42.1 \( p < 0.001 \) | 78                               |
|          | M     | 75   | 40.3 29 24.6–33.5 \( p < 0.001 \)   | 59                               |
|          | KRT86 | U   | 143 76.9 33 25.1–40.9 \( p < 0.001 \) | 64                               |
|          | M     | 43   | 23.1 26 18.1–33.1 \( p < 0.001 \)   | 73                               |
|          | RUNX3 | U   | 160 86.0 31 26.9–35.1 \( p < 0.001 \) | 73                               |
|          | M     | 26   | 14.0 21 4.7–37.2 \( p < 0.001 \)   | 55                               |
|          | CAMK2N1/RUNX3 | U   | 152 81.7 32 26.0–38.0 \( p < 0.001 \) | 74                               |
|          | M     | 34   | 18.3 21 3.8–38.1 \( p < 0.001 \)   | 57                               |
|          | CAMK2N1/KRT86 | U   | 133 71.5 37 25.9–48.1 \( p < 0.001 \) | 74                               |
|          | M     | 53   | 28.5 26 18.5–32.8 \( p < 0.001 \)   | 60                               |
|          | CAMK2N1/KRT86/RUNX3 | U   | 123 66.1 37 19.2–54.8 \( p < 0.001 \) | 74                               |
|          | M     | 63   | 33.9 29 23.0–35.0 \( p < 0.001 \)   | 60                               |
|          | BRCA1 | U   | 166 89.7 31 27.2–34.8 \( p < 0.001 \) | 73                               |
|          | M     | 19   | 10.3 25 11.8–37.4 \( p < 0.001 \)   | 57                               |

LogRank results with significance are marked in bold. U, unmethylated; M, methylated. Marker combinations are positive (methylated) if at least one single marker is methylated (<OR> combinations) otherwise the combination is scored as unmethylated.
Fig. 1  Kaplan–Meier plots for PFS and OS of sporadic EOC patients stratified by methylation markers. LogRank test was utilized for statistical evaluation.
prognostic effects related to overall survival. Only the combination of methylation markers CAMK2N1/RUNX3 exhibited a significant prognostic value for OS (Fig. 1, Table 2). The other methylation marker could not significantly differentiate between patient groups with varying OS (Fig. 1, Table 2). However, patients differentiated by CAMK2N1 methylation as singular marker exhibited a trend to varying overall survival ($p = 0.06$). Additionally, to the marker validation, we included methylation analyses for BRCA1. We could not identify any prognostic value for this parameter (Table 2) confirming data from the TCGA cohort and a recent meta-analysis [7, 53].

All methylation markers showing a significant prognostic value in univariate analysis (LogRank test) were separately analyzed together with both significant clinical parameters (FIGO and resection status) in multivariate Cox regression analysis (Table 3). We also analyzed clinical parameters with non-significant results in univariate analysis (histology, grading, treatment type) but they did neither exhibit prognostic value in multivariate analysis nor change results for the other clinical or molecular marker (data not shown). The combinations CAMK2N1/RUNX3 and CAMK2N1/RUNX3/KRT86 retained their significant prognostic value for PFS in this analysis. The calculated hazard ratio of progression for late stage EOC, after suboptimal debulking, for CAMK2N1/RUNX3 methylation and for CAMK2N1/RUNX3/KRT86 methylation was 5.029, 1.865, 1.587 and 1.543, respectively (Table 3). Thus, the above combinations of methylation markers had a prognostic value for PFS independent from FIGO staging and resection status. In relation to overall survival, the only the resection status and CAMK2N1/RUNX3 methylation showed an independent prognostic value (HR 2.45 and 1.60, respectively; Table 3). Late stage EOC exhibited a hazard ratio of 2.08 with $p = 0.051$.

### Explorative analysis of patients with defects in homologous recombination DNA repair (HRD)

A total of 10 patients with a familiar predisposition for EOC and a known mutation in BRCA1, BRCA2 or RAD51C and available tumor tissue were included in the cohort. The information of the exact mutation was reported by genetic counseling (Additional file 1: Table S1). Additionally, we screened young EOC patients with available tissue for an exploratory somatic BRCA1 mutation analysis and could identify five patients with mutations and included them in this cohort ($n = 15$, named HRD-defective EOC afterward, Additional file 1: Table S1).

Because of the small number of samples with mutations, all analyses have explorative character. We did not detect any significant differences in clinical variables between BRCAwt and HRD-defective EOC (data not shown). This is likely caused by the fact, that our cohort is enriched for high-grade serous EOC resembling BRCA1-mutated EOC. Only KRT86 showed significant different methylation frequencies in BRCAwt and HRD-defective EOC (22.9% and 0%, respectively; $p = 0.044$ Fisher's exact Test). A trend of similar differences was seen for CAMK2N1 and BRCA1 (8.5% and 11.2% methylated vs. absent methylation in BRCAwt vs. HRD-defective EOC, respectively). However, no significant difference was detected in the combined methylation frequency for all genes (60% of samples in both groups). Additionally, the prognostic value for PFS did not strongly differ between BRCAwt and HRD-defective EOC for most methylation marker although the small sample size precluded significant results (data not shown). Interestingly, we observed a difference in prognosis of patients with KATNAL2-methylated BRCAwt and HRD-deficient tumors compared to the respective unmethylated group. Because HRD-deficiency can also be potentially caused by BRCA1 methylation, we analyzed the prognostic value of KATNAL2

### Table 3 Results of multivariate Cox analyses of clinical and molecular variables

| Criteria | PFS | | OS | |
|----------|-----------------|-----------------|-----------------|-----------------|
|          | HR  | 95% CI  | P    | HR  | 95% CI  | P    |
| FIGO III/IV versus III/V ($n = 19$ vs $158$) | 5.029 | 2.029–12.46 | $0.001$ | 2.08 | 0.998–4.333 | $0.051$ |
| Resection R0 versus R1/2 ($n = 41$ vs $136$) | 1.865 | 1.257–2.766 | $0.002$ | 2.453 | 1.591–3.781 | $0.001$ |
| CAMK2N1 U versus M ($n = 162$ vs $15$) | 1.408 | 0.789–2.512 | $0.246$ | 1.300 | 0.670–2.524 | $0.438$ |
| KRT86 U versus M ($n = 138$ vs $39$) | 1.477 | 0.992–2.201 | $0.055$ | 1.243 | 0.787–1.964 | $0.351$ |
| CAMK2N1/KRT86 U versus M ($n = 129$ vs $48$) | 1.416 | 0.965–2.077 | $0.075$ | 1.214 | 0.788–1.871 | $0.379$ |
| CAMK2N1/RUNX3 U versus M ($n = 147$ vs $32$) | 1.587 | 1.031–2.442 | $0.036$ | 1.602 | 1.017–2.542 | $0.042$ |
| CAMK2N1/KRT86/RUNX3 U versus M ($n = 119$ vs $58$) | 1.543 | 1.070–2.223 | $0.020$ | 1.348 | 0.903–2.012 | $0.144$ |

Table 3: Results of multivariate Cox analyses of clinical and molecular variables. Analyses included clinical variables and one of the molecular markers. Data for clinical variables are exemplarily and result from the analysis with CAMK2N1/RUNX3. LogRank test results with significance are marked in bold. U, unmethylated; M, methylated. Marker combinations are positive (methylated) if at least one single marker is methylated (<OR> combinations) otherwise the combination is scored as unmethylated.
methylation in *BRCA*wt EOC with and without *BRCA1* methylation and HRD-deficient EOC (Fig. 2). Whereas *KATNAL2* methylation showed no prognostic effect in the complete cohort (*n* = 188, Table 2) the stratification into *BRCA* aberration dependent groups resulted in significant prognostic effects (*p* < 0.05, LogRank test). *KATNAL2* methylation correlated with a significant worse outcome in patients with *BRCA*wt, but with a prolonged PFS in HRD-deficient and *BRCA1*-methylated EOC patients compared to the patients with respective *KATNAL2* unmethylated tumors (29 vs. 37 month (*p* = 0.012, HR 1.591), 35 vs. 31 month (n.s., HR 0.620) and 32 vs. 19 month (*p* = 0.04, HR 0.332), respectively; Fig. 2).

**Discussion**

Aberrant DNA methylation can be used as molecular biomarker for disease diagnosis, prognosis or therapy stratification [58, 59]. Importantly, both the specific use and the target population must be defined and validated. Biomarker establishment and validation must balance between the power of the marker, the quality of the results and the homogeneity of the target population. In previous work, we identified 5 methylation markers that were prognostic for PFS by univariate analysis in a defined population—late stage type II EOC treated with platinum-taxane-based chemotherapy [44]. The aim of the present study was to analyze the potential independent prognostic value of these markers for PFS and OS in a group of EOC patients more heterogeneous in regard to tumor stage, grading, tumor histology and treatment by multivariate analyses. To provide excellent sample quality and correct sample classification, pathological evaluation of tissues was done. Because samples originated from two different medical centers, handling differences cannot be excluded, but the processing (cryo-sectioning, DNA purification), bisulfite conversion and MSP was done in one laboratory only. Moreover, the robustness of methylation analyses was determined by repeated assays from selected patients using different tissue blocks of the same tumor or repeated sections from identical biopsies resulting in an almost perfect agreement (Cohen’s kappa 0.881). We included samples with tumor cell fraction >10% and did not observe a strong influence of the tumor content on methylation frequency of candidate regions (Additional file 1: Fig. S3). This may relate to the low cut-off value used to assign a positive methylation status (1% relative methylation, see method section) and absent methylation in normal keratinocytes, PBMCs and whole tissue sections of normal ovaries (data not shown). However, several limitations prohibit the transfer of the presented results to the general EOC population. First of all, the analyzed cohort does not represent all histological subtypes. Secondly, both median progression-free and overall survival are longer for our cohort compared to the general EOC population in Germany (31 month vs. 18.2 month PFS, 73 month vs. 44.1 month OS; [60]). The 5-year survival rate of 56% in our cohort is higher than the population-based rate in Germany (41%) [1]. Reasons for this are (1) the selection of platin-sensitive EOC and (2) the high proportion of optimal resected patients (macroscopically tumor-free, ~80%). A third limitation is that only a subset of patients had available information about germline mutation status (*n* = 10). An additional exploratory somatic *BRCA1* mutational analysis was done in early onset patients (age at diagnosis < 40 years) and revealed 5 patients with *BRCA1* mutations. Recent studies in Germany detected an approximately 20% *BRCA* germline mutation rate in unselected EOC patients (Harter [6]) suggesting that we underestimate the HRD-deficient fraction in our cohort (15/203, 7.4%).

![Fig. 2](image-url) **Fig. 2** Kaplan–Meier plots of PFS for patients with sporadic EOC without *BRCA1* methylation (*BRCA*wt), EOC with mutations in HRD genes (*BRCA* mut) or sporadic EOC with *BRCA1* methylation (*BRCA1*meth) stratified by *KATNAL2* methylation.
Nevertheless, this may only affect the prognostic value of \textit{KATNAL2} (see below) because other markers seem to be not affected by HRD-status.

Using this enlarged, more heterogeneous cohort of EOC patients (Table 1) we could confirm the prognostic value of singular markers (\textit{CAMK2N1}, \textit{KRT86}) and combinations (\textit{CAMK2N1/RUNX3}; \textit{CAMK2N1/RUNX3/KRT86}) for PFS in univariate analysis. Additionally, both combinations are prognostic independent from tumor stage and resection status (multivariate Cox regression analysis). The stronger effect of combinations is not caused by the pure combination of markers potentially resulting in the detection of EOC with generally higher methylation level because EOC unmethylated for all analyzed marker showed no significant different survival compared to the group of EOC with at least one methylated marker (Table 2). It is more likely that informative methylation markers detect specific but different subgroups of EOC with reduced survival. This is supported by the low methylation frequency of informative markers (\textit{CAMK2N1} 7.9\%, \textit{KRT86} 22.3\%, \textit{RUNX3} 14.9\%). Whereas \textit{CAMK2N1} and \textit{RUNX3} methylation is independent from FIGO staging, \textit{KRT86} methylation is not an independent prognostic factor but associated with late stage EOC. Thus, \textit{KRT86} methylation can potentially be used for pre-operative staging if it can be specifically detected in systemic samples, e.g., blood. Besides the prognostic effect for PFS we could also detect an association of the marker combination \textit{CAMK2N1/RUNX3} with overall survival both in univariate and multivariate analysis. EOC patients with methylation of \textit{CAMK2N1} or \textit{RUNX3} have a shorter overall survival than patients without methylation of these genes independently from FIGO staging or tumor resection status. The effect on overall survival is mainly caused by the shorter PFS in \textit{CAMK2N1/RUNX3} methylation positive EOC patients (Fig. 1, Table 2) as Kaplan–Meier analyses for the time interval between relapse and death or last information available did not show a prognostic value for the marker combination (subset of relapsed patients $n = 133$ (70.7\%); data not shown).

If prospectively validated \textit{CAMK2N1/RUNX3} methylation may identify a subgroup of platin-sensitive patients that have worse prognosis. It can be speculated that this subgroup may benefit from maintenance treatment with Bevacizumab because both GOG-218 and ICON7 identified an overall survival benefit in a group of patients with high risk for progression differently defined by clinical variables [61, 62]. Although 29 patients of our cohort were treated with Bevacizumab, the number of methylation events ($n = 2$) is too low to draw any conclusion. However, published and unpublished data point to an influence of \textit{RUNX3} on the angiogenic potential of EOC by influencing the expression of thrombospondin-1 and other angiogenic factors ([63, 64], Heinze et al. in preparation). We also tried to identify an association of methylation marker status with \textit{BRCA} status (\textit{BRCA} mutation, \textit{BRCA1} methylation) but did not detect a largely different prognostic value of \textit{CAMK2N1/RUNX3} methylation in \textit{BRCAwt} and potentially HRD-deficient EOC. Nevertheless, \textit{KATNAL2} methylation being not prognostic in the complete group of EOC showed opposite effects in \textit{BRCAwt} EOC without \textit{BRCA1} methylation (HR 1.591, $p = 0.012$) and \textit{BRCAwt} EOC with \textit{BRCA1} methylation (HR 0.332, $p = 0.04$) or \textit{BRCAwt} EOC (HR 0.620, n.s.). Although the numbers of patients with either \textit{BRCA} aberration are small, this suggests different consequences of \textit{KATNAL2} methylation in \textit{BRCAwt} versus HRD-deficient EOC. Shared clinico-pathological features between \textit{BRCA1}-mutated and \textit{BRCA1}-methylated tumors may relate to the inactivation of \textit{BRCA1} albeit only \textit{BRCA1} mutations significantly influence patients survival [53]. Likely, we did not identify a prognostic value of \textit{BRCA1} methylation (Table 2). For patients with \textit{BRCA1}-mutated tumors, survival analyses are limited by the low number and the shorter follow-up period for these patients (median mutated vs. wt: 36 month vs. 55 month). The PFS was not significantly different in \textit{BRCA1}-mutated and \textit{BRCA1}wt patients in our cohort (data not shown). In preliminary experiments, we analyzed \textit{BRCA1} protein expression by immunohistochemistry in a subset of samples and could observe significantly different staining patterns between \textit{BRCA1}wt and mutated or methylated tumors (Additional file 1: Table S3) supporting earlier results from methylated tumors [52]. Both groups of tumors with \textit{BRCA1} aberration express lower levels of \textit{BRCA1}. Thus, loss of \textit{BRCA1} may indeed result in the switch of the prognostic value of \textit{KATNAL2} methylation. \textit{KATNAL2} (Katanin Catalytic Subunit A1 Like 2) is a gene coding for a protein with microtubule binding and microtubule-severing activity whose detailed function in carcinogenesis is largely unknown. However, methylation of \textit{KATNAL2} is a potential marker for severe cervical intraepithelial neoplasia [65]. The \textit{Katanin} gene family regulates microtubule dynamics during mitosis, migration, ciliogenesis or cellular reorganization [66]. Strong \textit{KATNAL2} downregulation leads to massive cell death, whereas mild downregulation causes microtubule stabilization and reduced turnover leading to mitotic defects and G2/M arrest in mouse cells [67]. Therefore, it can be speculated that microtubule stabilization by methylation-dependent \textit{KATNAL2} downregulation induces mitotic defects, chromosome missegregation and chromosomal instability (CIN) potentially enabling tumor adaptation to chemotherapeutic intervention and worse outcome in \textit{BRCA1}wt patients. However, \textit{BRCA} proteins
are not only involved in DNA repair but also microtubule organization—specifically in mitosis and cell polarization [68, 69]. Importantly, BRCA1 loss results in disturbed mitosis and CIN [70]. Thus, in tumors with BRCA1 aberrations, the sum of mitotic defects may result in mitotic catastrophe, increased tumor cell death/better therapeutic response and improved survival. Besides the unknown basic mechanism for the different prognostic impact of KATNAL2 methylation, depending on HRD status, this points to the potential necessity to differentiate between these EOC groups for biomarker identification and validation studies.

New biomarker candidates for cancer are identified frequently, a small subset of them will be published in peer-reviewed journals and only a few are validated in independent studies. However, even a positive validation does not guarantee a successful biomarker candidate [71]. Only ~ 0.8% of published methylation markers are commercially available [72]. Additional knowledge of the underlying mechanism of the biomarker’s detection and function would be helpful to underline its significance. One validation step for DNA methylation marker can consist of immunohistochemical detection of the epigenetically regulated protein. However, DNA methylation may not result in largely different expression a priori but rather restricts the capacity for an increased expression. Such regulation of the expression potential of affected genes was observed for genes hypermethylated during mitosis and CIN [70]. Thus, in tumors with BRCA1 aberrations, the sum of mitotic defects may result in mitotic catastrophe, increased tumor cell death/better therapeutic response and improved survival.

In the present study, we could not confirm a significant prognostic value of singular RUNX3 methylation but a trend for decreased PFS for patients with tumors showing methylated RUNX3 (median PFS 21 month vs. 31 month; \( p = 0.1 \) LogRank test).

**Conclusion**

In conclusion, the retrospective analysis of 188 sporadic platinum-sensitive EOC proved an independent prognostic value of the methylation marker combination CAMK2NI/RUNX3 for PFS and OS. If validated prospectively this combination may identify EOC patients with worse prognosis after standard therapy potentially benefiting from intensive follow-up, maintenance therapies or inclusion in therapeutic studies.

**Methods**

**Patients material**

A total of 203 fresh frozen (FF) and formalin-fixed paraffin-embedded (FFPE) tissue samples were used and divided into a validation and BRCA mutation cohort. The samples originated from the University Medical Center Hamburg-Eppendorf and the Jena University Hospital from 1996 to 2016. The use of patient’s material was approved by the Ethics committee Jena (#2582-6/09) and Hamburg (#190505). Clinical data were retrieved from the medical records. Progression-free survival was calculated from the date of primary surgery to first occurrence of relapse (confirmed by second-look surgery or non-invasive diagnostic tools) or last follow-up and overall survival was calculated from date of primary surgery until death or last follow-up. Surgical samples not needed for diagnostic procedures were snap-frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\). The samples for the validation set were included based on the existence of three-years-follow-up data. Detailed and summary information on those two cohorts are depicted in Table 1 and Additional file 1: Table S1.

Hematoxylin–Eosin stained sections of analyzed tissues were evaluated for histopathological classification and

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Hematoxylin–Eosin stained sections of analyzed tissues were evaluated for histopathological classification and
estimation of tumor cell fraction. Only tissues with >10% tumor cells were used and 24.6%, 44.2% and 22.8% of samples contained 10–40%, 40–80% and >80% tumor cells.

**DNA isolation**
Sections from FF and FFPE tissue were used to perform analysis. The isolation was done according to the phenol-chloroform-isoamyl alcohol (PCI)-based DNA isolation protocol and using the QIAamp Kit according to the manufacture instruction.

**RNA isolation and cDNA synthesis for BRCA mutation detection**
Sections from selected FF tissue were used for RNA isolation and subsequent cDNA synthesis. The isolation was done with the NucleoSpin® RNA kit according to the manual (Macherey–Nagel). 500 ng of RNA was reverse transcribed according to established techniques following published protocols [56].

**Bisulfite conversion**
500 ng–1 µg of genomic DNA derived from tissue was converted using the EZ DNA Methylation-Gold™ according to the manual instruction (Zymo Research). The bsDNA concentration was determined and adjusted to 5 ng/µl. Identically treated gDNA from cervical swab of a healthy individual served as negative control sample. Positive control sample was created by in vitro methylation using the CpG-Methyltransferase (M.SssI), according to the supplier’s instruction (New England BioLabs). A serial dilution using in vitro methylated and unmethylated control bsDNA was created to generate the standard of 0%, 1%, 10% and 100% methylation level.

**Methylation-specific PCR**
The q-MSP was performed according to [44] using the RotorGeneQ thermocycler (Qiagen, Germany) and candidate gene-specific primer listed in Additional file 1: Table S2 [44, 52]. All MSP assays detect DNA methylation within CpG islands located in the promoter/first exon region of associated genes except KRT86. The analyzed CpG island for KRT86 is located at the 3’end (last exon) of the gene. Melting curve analyses confirmed the specificity of MSP amplification. The level of methylation was quantified stepwise by (1) calculating the relative amount (2ΔCt, relative methylation) of methylated target sequences in relation to a beta-actin fragment which was amplified by bisulfite-DNA-specific, but methylation-independent PCR (BS-PCR) and (2) comparing the relative methylation to an artificial dilution series (0–100% methylated DNA). Methylation data were evaluated qualitatively by application of a cutoff value and only samples showing a higher relative methylation as the 1% control were scored as methylated. Marker combinations reflect an <OR> combination of single marker results.

**BRCA1 mutation detection**
To detect BRCA1 exon mutations, a total of 6 PCRs were done using cDNA derived from patient’s RNA. Approximately 20 ng reverse transcribed nucleic acid were used. The reactions were conducted in 50 µl volume containing: dNTPs (240 µM each), forward and reverse primer (10 pmol each), DMSO (5%), MgCl2 (1.75 mM), AmpliTaqGold (1.25 U) and respective reaction buffer II (Applied Biosystems). The PCR steps were as follows: initial denaturation and activation at 95 °C for 10 min followed by 25 cycles of denaturation phase at 95 °C for 15 s, primer-specific annealing for 20 s at 56 °C and elongation at 72 °C for 30 s. The reaction products were size separated and sequenced in both directions. The readings were aligned to the BRCA1 sequence according to human reference sequence (hg19).

**Statistical analysis**
The statistical analysis was executed using SPSS and Microsoft Excel 2010/365. The unpaired student’s t-test was used for pairwise comparisons of continuous variables whereas chi-squared or Fishers Exact test was used for ordinal variables. The difference in survival between groups of patients was depicted by Kaplan–Meier plots and evaluated by LogRank test. Multivariate Cox regression analyses were used to calculated hazard ratios and determine the independent prognostic value of methylation markers.

**Supplementary information**
The online version contains supplementary material available at https://doi.org/10.1186/s13148-021-01006-8.

**Additional file 1:** Figure S1. Graphical overview of methylations-specific PCR validation results. Figure S2. Kaplan–Meier plots for PFS and OS of EOC patients stratified by clinical variables. Figure S3. Comparison of methylation frequency in tumors with different tumor cell fraction. Table S1. Clinical data and mutation data of EOC with known mutations (n = 15). Table S2. Primer data for MSP and BRCA1 mutation analysis. Table S3. BRCA1 immunohistochemistry results for a subset of tumors depending on BRCA1 aberrations.

**Abbreviations**
Carbo: Carboplatin; DNMT: DNA-methyl-transferase; DNA: Deoxyribonucleic acid; EOC: Epithelial ovarian cancer; FIGO: International Federation of Gynecology and Obstetrics; HBOC: Hereditary breast and ovarian cancer syndrome; HGSOC: High-grade serous ovarian cancer; HRD: Homologous DNA repair defects; M: Methylated; MBD: Methyl-binding domain protein; MSP: Methylation-specific PCR; OS: Overall survival; Pac: Paclitaxel; PARP: Poly-ADP-ribose polymerase; PARPi: PARP inhibitor; PFS: Progression-free survival; U: Unmethylated.
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Authors’ contributions
Conceptualization: MD, NH; IBR. Data curation: KH, NH; Funding acquisition: NH; Investigation: KH, MG, LJ, LO, NH; Methodology: NH; Project administration: MD, NH, IBR. Material and Resources: MR, LOF, BS, IBR. Supervision: MD, NH; Writing—original draft: KH, NH; Writing—review and editing: MR, MG, LJ, LO, LOF, BS, IBR. All authors approved the final manuscript version. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article [and its supplementary information files]. Raw data are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The use of patient’s material and clinical data was approved by the Ethics committee Jena (#2582-6/09) and Hamburg (#190505).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
1 Department of Gynecology and Reproduction Medicine, Jena University Hospital—Friedrich Schiller University Jena, 07747 Jena, Germany. 2 Department of Forensic Medicine, Section of Pathology, Jena University Hospital – Friedrich Schiller University Jena, 07747 Jena, Germany. 3 Department of Gynecology, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany.

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References
1. PKI, GEKD. Krebs in Deutschland 2013/2014. Berlin: Robert-Koch-Institut; 2017. p. 88–91.
2. Lynch HT, Casey MJ, Snyder CL, et al. Hereditary ovarian carcinoma: heterogeneity, molecular genetics, pathology, and management. Mol Oncol. 2009;3(2):97–137.
3. Kobayashi H, Ohno S, Sasaki Y, et al. Hereditary breast and ovarian cancer susceptibility genes (review). Oncol Rep. 2013;30(3):1019–29.
4. Nielsen FC, van Overeem HT, Sorensen CS. Hereditary breast and ovarian cancer: new genes in confined pathways. Nat Rev Cancer. 2016;16(9):599–612.
5. Norquist BM, Harrell MI, Brady MF, et al. Inherited mutations in women with ovarian carcinoma. JAMA Oncol. 2016;2(4):482–90.
6. Harter P, Hauke J, Heitz F, et al. Prevalence of deleterious germline variants in risk genes including BRCA1/2 in consecutive ovarian cancer patients (AGO-TR-1). PLoS ONE. 2017;12(10):e0186043.
7. Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian cancer. Nature. 2011;474(7353):609–15.
8. Hauke J, Hennen E, Schneider S, et al. Deleterious somatic variants in 473 consecutive individuals with ovarian cancer: results of the observational AGO-TR1 study (INCT02222893). J Med Genet. 2019;56(9):574–90.
9. Vaughan S, Coward JL, Bast RC Jr, et al. Rethinking ovarian cancer: recommendations for improving outcomes. Nat Rev Cancer. 2011;11(10):719–25.
10. Rescigno F, Cerillo I, Ruocco R, et al. New hypothesis on pathogenesis of ovarian cancer lead to future tailored approaches: Biomed Res Int. 2013;2013:852839.
11. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. CA Cancer J Clin. 2017;67(1):7–30.
12. Sorensen SM, Schnack TH, Högdall C. Impact of residual disease on overall survival in women with Federation of Gynecology and Obstetrics stage III/IV epithelial ovarian cancer after primary surgery. Acta Obstet Gynecol Scand. 2019;98(1):34–43.
13. du Bois A, Reuss A, Pujade-Lauraine E, et al. Role of surgical outcome as prognostic factor in advanced epithelial ovarian cancer: a combined exploratory analysis of 3 prospectively randomized phase 3 multicenter trials: by the Arbeitsgemeinschaft Gynaekologische Onkologie Studiengruppe Ovarialkarzinom (AGO-OVAR) and the Groupe d’Investigateurs Nationaux Pour les Etudes des Cancers de l’Ovaire (GINECO). Cancer. 2009;115(6):1234–44.
14. Greenlee RT, Hill-Harmon MB, Murray T, et al. Cancer statistics, 2001. CA Cancer J Clin. 2001;51(1):15–36.
15. Davis A, Tinker AV, Friedlander M. “Platinum resistant” ovarian cancer: what is it, who to treat and how to measure benefit? Gynecol Oncol. 2014;133(5):624–31.
16. Bolton KL, Chenevix-Trench G, Goh C, et al. Association between BRCA1 and BRCA2 mutations and survival in women with invasive epithelial ovarian cancer. JAMA. 2012;307(4):382–90.
17. McLaughlin JP, Rosen B, Moody J, et al. Long-term ovarian cancer survival associated with mutation in BRCA1 or BRCA2. J Natl Cancer Inst. 2013;105(2):141–8.
18. Ledermann J, Harter P, Gourley C, et al. Olaparib maintenance therapy in patients with platinum-sensitive relapsed serous ovarian cancer: a preplanned retrospective analysis of outcomes by BRCA status in a randomised phase 2 trial Lancet Oncol. 2014;15(8):852–61.
19. Mirza MR, Monk BJ, Herrstedt J, et al. Niraparib maintenance therapy in platinum-sensitive, recurrent ovarian cancer. N Engl J Med. 2016;375(2):154–64.
20. Hill SJ, Decker B, Roberts EA, et al. Prediction of DNA repair inhibitor response in short-term patient-derived ovarian cancer organoids. Cancer Discov. 2018;8(11):1404–21.
21. Gentles L, Goranov B, Matheson E, et al. Exploring the frequency of homologous recombination DNA repair dysfunction in multiple cancer types. Cancers (Basel). 2019;11(3):334.
22. Gonzalez-Martin A, Pothuri B, Vergote I, et al. Niraparib maintenance therapy in platinum-sensitive, recurrent ovarian cancer: recommendations for improving outcomes. Nat Rev Dis Primers. 2016;2:16061.
23. Amante DB, Calape L, Ziman M, et al. Liquid biopsy in ovarian cancer using circulating tumor DNA and cells: ready for prime time? Cancer Lett. 2020;468:59–71.
24. Man R, Mamesier E, Lambaudie E, et al. Liquid biopsies for ovarian carcinoma: how blood tests may improve the clinical management of a deadly disease. Cancers (Basel). 2019;11(6):774.
25. Sheng R, Li X, Wang Z, et al. Circular RNAs and their emerging roles as diagnostic and prognostic biomarkers in ovarian cancer. Cancer Lett. 2020;473:139–47.
26. Zhu Y, Zhou S, Liu Y, et al. Prognostic value of systemic inflammatory markers in ovarian cancer: a PRISMA-compliant meta-analysis and systematic review. BMC Cancer. 2018;18(1):443.
27. Frawley T, Piskareva O. Extracellular vesicle dissemination of epidermal growth factor receptor and ligands and its role in cancer progression. Cancers (Basel). 2020;12(11):3200.
28. Drakes ML, Stiff PJ. Regulation of ovarian cancer prognosis by immune cells in the tumor microenvironment. Cancers (Basel). 2018;10(9):302.
29. Etemadmoghadam D, deFazio A, Beroukhim R, et al. Integrated genome-wide DNA copy number and expression analysis identifies distinct mechanisms of primary chemoresistance in ovarian carcinomas. Clin Cancer Res. 2009;15(4):1417–27.
31. Etemadmoghadam D, Weir BA, Au-Yeung G, et al. Synthetic lethality between CCNE1 amplification and loss of BRCA1. Proc Natl Acad Sci USA. 2013;110(48):19489–94.
32. Konieczny GE, Winterhoff B, Wang G. Gene-expression signatures in ovarian cancer: promise and challenges for patient stratification. Gynecol Oncol. 2016;141(2):379–85.
33. Samuel P, Carter DR. The diagnostic and prognostic potential of microRNA-mutations in epithelial ovarian carcinoma. Mol Diagn Ther. 2017;21(1):59–73.
34. Barton CA, Hacker NF, Clark SJ, et al. DNA methylation changes in ovarian cancer: implications for early diagnosis, prognosis and treatment. Gynecol Oncol. 2008;109(1):129–39.
35. Gifford G, Paul J, Valsey PA, et al. The acquisition of hMLH1 methylation in DNA. Plasma after chemotherapy predicts poor survival for ovarian cancer patients. Clin Cancer Res. 2004;10(13):4420–6.
36. Michaelson-Cohen R, Keshet I, Straussman R, et al. Genome-wide de novo methylation in epithelial ovarian cancer. Int J Gynecol Cancer. 2011;21(2):269–79.
37. Patch AM, Christie EL, Etemadmoghadam D, et al. Whole-genome characterization of chemoresistant ovarian cancer. Nature. 2015;521(7553):489–94.
38. Hafner N, Steinbach D, Jansen L, et al. RUNX3 and CAMK2N1 hypermethylation as prognostic marker for epithelial ovarian cancer. Int J Cancer. 2016;138(1):217–28.
39. Ibanez de Caceres I, Battaglia C, Esteller M, et al. Tumor cell-specific BRCA1 and RASSF1A hypermethylation in serum, plasma, and peripheral blood from ovarian cancer patients. Cancer Res. 2004;64(18):6476–81.
40. Arnold JM, Cummings M, Purdie D, et al. Reduced expression of intercellular adhesion molecule-1 in ovarian adenocarcinomas. Br J Cancer. 2001;85(9):1351–8.
41. Yuecheng Y, Hongmei L, Xiaoyan X. Clinical evaluation of E-cadherin and RASSF1A expression as markers for predicting chemoresistance in epithelial ovarian cancer. Oncol Lett. 2019;23(6):2517–27.
42. Arnold JM, Cummings M, Purdie D, et al. Reduced expression of intercellular adhesion molecule-1 in ovarian adenocarcinomas. Br J Cancer. 2001;85(9):1351–8.
43. Tewari KS, Burger RA, Enserro D, et al. Final overall survival of a randomized trial of bevacizumab for primary treatment of ovarian cancer. J Clin Oncol. 2019;37(26):2317–20.
44. Shi X, Deepak V, Wang L, et al. Thrombospondin-1 is a putative target gene of RUNX2 and RUNX3. Int J Mol Sci. 2013;14(7):14321–32.
45. Pfisterer J, Oza AM, Cook AD, et al. Standard chemotherapy with or without bevacizumab for newly diagnosed ovarian cancer (ICON7): overall survival results of a phase 3 randomised trial. Lancet Oncol. 2015;16(8):928–36.
46. Radosa MP, Hafner N, Camara O, et al. Loss of BRCA1 protein expression as an indicator of the BRCAness phenotype is associated with favorable overall survival after complete resection of sporadic ovarian cancer. Cancer Res. 2011;71(2):649–57.
47. Yang Z, Liu Y. DNA methylation in human diseases. Genes Dis. 2018;5(1):376–92.
48. Jones PA, Baylin SB. The epigenomics of cancer. Cell. 2007;128(4):683–92.
49. Hafner N, Steinbach D, Jansen L, et al. RUNX3 and CAMK2N1 hypermethylation as prognostic marker for epithelial ovarian cancer. Int J Cancer. 2016;138(1):217–28.
50. Koch A, Joosten SC, Feng Z, et al. Analysis of DNA methylation in cancer: location revisited. Nat Rev Clin Oncol. 2018;15(7):459–66.
51. Ververis A, Christodoulou A, Christoforou M, et al. A novel family of katin-like 2 protein isoforms (KATNAL2), interacting with nuclear-binding proteins Nubp1 and Nubp2, are key regulators of different MT-based processes in mammalian cells. Cell Mol Life Sci. 2016;73(13):1633–43.
52. Kuo YW, Howard J. Cutting, amplifying, and aligning microtubules with severing enzymes. Trends Cell Biol. 2020;30(1):50–61.
53. Koch A, Joosten SC, Feng Z, et al. Analysis of DNA methylation in cancer: location revisited. Nat Rev Clin Oncol. 2018;15(7):459–66.
54. Arnold JM, Cummings M, Purdie D, et al. Reduced expression of intercellular adhesion molecule-1 in ovarian adenocarcinomas. Br J Cancer. 2001;85(9):1351–8.
55. Radosa MP, Hafner N, Camara O, et al. Loss of BRCA1 protein expression as an indicator of the BRCAness phenotype is associated with favorable overall survival after complete resection of sporadic ovarian cancer. Cancer Res. 2011;71(2):649–57.
56. Radosa MP, Hafner N, Camara O, et al. Loss of BRCA1 protein expression as an indicator of the BRCAness phenotype is associated with favorable overall survival after complete resection of sporadic ovarian cancer. Cancer Res. 2011;71(2):649–57.
83. Gu H, Gu S, Zhang X, et al. miR-106b-5p promotes aggressive progression of hepatocellular carcinoma via targeting RUNX3. Cancer Med. 2019;8(15):6756–67.
84. Fan L, Wang Y, Huo W, et al. MicroRNA-301a-3p overexpression promotes cell invasion and proliferation by targeting runt-related transcription factor 3 in prostate cancer. Mol Med Rep. 2019;20(4):3755–63.
85. Zhao HZ, Zhao HL, Zhang YN, et al. MicroRNA-199b promotes cell proliferation and invasion in Wilms' tumour by directly targeting Runt-related transcription factor 3. Mol Med Rep. 2018;18(2):1812–9.

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