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Identifying Novel Targetable Chromosomal Alterations in Ovarian Cancer: Using Germline Copy Number Variation Association Analysis

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ABSTRACT

The heterogeneity of ovarian cancer (Ov Ca) is attributed to multiple genetic and epigenetic changes, rendering it difficult to detect the most relevant molecular alterations. Identifying copy number variations (CNVs) will be helpful in screening patients with a familial history and will ultimately facilitate early diagnosis. This work aims to determine germline CNVs that may be associated with risks for different subtypes of ovarian cancer. Using Affymetrix genome-wide human SNP 6.0 arrays, 138 germline DNA samples of non-familial ovarian cancer were analysed using Golden Helix (SVS7) software. CNVs overlapping the EYA2 (20q13.12) and WNK1 (12p13.33) genes are the top hits with a significant p-value (<0.05). Deletion is more frequent in normal and low-grade carcinomas. Commonly, ovarian cancer is copy neutral (CN2) or has copy number gains (CN3). Amplification at these locations is associated with high-grade cases, which have worse overall survival rates. A CN3 in the WNK1 gene is associated with a higher expression of mRNA. It could be concluded that ovarian cancer is associated with CN3s where the segments of DNA overlap WNK1 and EYA2. The oncogenic effect of WNK1 and EYA2 on ovaries may serve as prognostic markers for ovarian cancer.

KEYWORDS

Copy number variations; EYA2; ovarian cancer; WNK1

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1. Introduction

Ovarian carcinoma (Ov Ca) is the seventh most common malignancy in women. It is a major public health problem, as there are more than 190,000 new cases of Ov Ca worldwide every year, with multiple etiologic hypotheses for ovarian carcinogenesis. There are neither applicable biomarkers to ascertain early disease nor consistent prognostic markers for predicting clinical response to treatment (Concolino *et al.*, 2018; Siegel *et al.*, 2019; Webb and Jordan, 2017). The heterogeneity of Ov Ca leads to a histopathologic subtype that carries distinct genetic events with complex genomic alterations. Hence, the importance of recognising disease diversity has clinical implications for the strategies of early detection, prevention and treatment (Alexandrova *et al.*, 2020; Kossai *et al.*, 2018).

Copy number variations (CNVs) are duplications or deletions of several kilo-base or more segments of nuclear DNA. CNVs are associated with the risk of developing common diseases, including cancers (Concolino *et al.*, 2018; C. Lee *et al.*, 2007). The recent application of genotyping platforms reliably detects copy number information that serves as another intense basis of genetic variability to outline complex molecular and biochemical interactions within the human genome (C. Lee *et al.*, 2007).

Eyes absent (*EYA*), homologous to the Drosophila eyes absent genes, form a family of genes first identified in Drosophila and other organisms (mice, humans, molluscs, nematodes). *EYA2*, mapped to chromosome 20q13 and *EYA* mutations, are associated with multi-organ birth defects in humans (Zhang *et al.*, 2005). *EYA*s are required for the development of multiple organs and have correspondingly been implicated in multiple disorders. Increasing evidence points to an oncogenic, pro-metastatic and angiogenic function for *EYA*. *EYA2* functions as a transcriptional coactivator to activate a variety of target genes involved in cell cycle progression and differentiation in several tissues (Tadjuidje and Hegde, 2013). Overexpression of *EYA*s occurs in tumorigenesis and metastasis processes by working on the tumour microenvironment to activate tumour cell growth. *EYA2* mRNA is highly expressed in most human cancers, such as ovarian, prostate, lung, breast and urinary tract, while its expression is low in colon cancer (Liu *et al.*, 2019; Tadjuidje and Hegde, 2013).

*WNK*s (with no lysine [K]) express four isoforms belonging to the serine/threonine kinases family of genes (Xu *et al.*, 2000). *WNK* family proteins play an integral role in cell proliferation, differentiation and carcinogenesis (Moniz and Jordan, 2010). *WNK1* is located in Chr12p13.33, and allelic losses in the Chr12p12.2-p13 region that harbours *WNK1* are in breast and ovarian cancers. *WNK1* point mutations are associated with breast, prostate, ovarian, colorectal, lung and thyroid cancers, which confirms its potential role in tumorigenesis and metastasis formation (Kori and Yalcin Arga, 2018; Rodan and Jenny, 2017).

CNVs could play a substantial role in conferring Ov Ca. This study aims to identify germline CNVs that may be associated with risks for different subtypes of Ov Ca. The use of a genome-wide copy number analysis-testing platform would enable the identification of novel and potentially targetable chromosomal alterations of therapeutic significance.

2. Materials and Methods

2.1. Cohort:

The samples consist of 138 germline DNA of non-familial ovarian cancer drawn from a CA-125 screened cohort of individuals with peripheral blood samples from 1996, which was a collaborative work between Harvard Medical School and the Chinese University of Hong Kong. The cohort is composed of ethnically homogeneous women

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(Chinese descent) obtained from an ethnically homogeneous population to avoid detection of CNVs related to differences in ethnicity and not to an ovarian cancer genetic signature. The study population is aged 15–94 years (mean=49.8) and presents with different pathological subtypes of Ov Ca. The Ov Ca cases in this cohort are divided into three groups based on age: the smallest group involves cases younger than 21 years (1.5%), the second ranges between 21–45 years (22%) and the most common group consists of patients older than 45 (76.5%). Most of the cases are serous carcinoma subtypes (31.1%), followed by endometrioid carcinoma (20.5%), then clear cell carcinoma (12.9%), while other types represent 34.4%. Overall survival curves in this cohort show that the worst survival rate is the malignant mixed Mullerian tumour (MMMT), followed by adenocarcinomas and serous carcinomas, while maximum survival is detected in clear cell carcinoma and endometrioid carcinoma. Tumour grades are classified into a low grade (63%) and high grade (37%), and the cases comprise stage I (38.5%), stage II (15.4%), stage III (36.9%) and stage IV (9.2%). Normal controls (30 samples) are from an ethnically homogeneous population to avoid the detection of CNVs related to differences in ethnicity and not related to an ovarian cancer genetic signature.

All procedures implemented in this study have been approved by IRB and/or the national research ethics committee in agreement with the 1964 Helsinki Declaration and its later amendments.

2.2. Study Outline:

Affymetrix genome- wide human SNP 6.0 arrays

DNA from 138 Ov Ca cases belonging to different grades, stages and subtypes with Affymetrix genome-wide human SNP 6.0 arrays were analysed using Affymetrix Genotyping Console (GTC) software to generate copy number, loss of heterozygosity (LOH) and segmentation data from the raw intensity values (CEL files). All cases were processed according to the manufacturer's protocol (Affymetrix Santa Clara, CA, USA).

Affymetrix GTC generates copy number and segmentation data using the Birdseed v2 algorithm. Golden Helix software (SVS7) provides advantages in correcting the batch effect as well as detecting the t-wave effect to minimise noisiness and eliminate false-positive results. The software suite also has a module to carry out association analysis. Segmentation algorithms convert the log-ratio data to copy number segments to yield a list of inherited and de novo CNVs on a univariate and multivariate basis. CNVs that did not have a significant p-value for association analysis between cancer and normal groups were excluded. The results provide heat map pictures of multiple genetic loci of lesions associated with Ov Ca and provide avenues for further identification of oncogenes and tumour suppressor genes involved in Ov Ca.

2.3. Copy Number Validation:

TaqMan DNA copy number validation by qPCR

DNA from 138 ovarian cancer cell lines were used for TaqMan quantitative polymerase chain reaction (qPCR; Life Technologies, CA) validation in addition to human and HapMap samples. HapMap cell lines NA10851 and NA12878 (Coriell Institute) were used as references for qPCR validation. DNA was extracted from all cell lines using Puregene DNA Purification Kit (Gentra Systems). Copy Caller software was used to analyse the copy number experiments provided by Applied Biosystems.

The genomic coordinates of the top hits from the Golden Helix analysis were extracted from the association analysis table. The copy number states of the top CNVs were validated using qPCR. TaqMan qPCR was preferred because it has a more reliable primer design, which is more specific and sensitive. Each experiment was repeated three times, using three replicates of the same sample in each run to ensure consistency of results.

2.4. NanoString Validation:

NanoString nCounter analysis techniques (NanoString Technologies, WA) were performed to confirm the results detected by association analysis. NanoString technology was used to further confirm the copy number state of a specific DNA segment that was seen to be significantly associated with serous ovarian cancer.

For NanoString analysis, samples representing different copy number states of two specific CNV segments previously identified by Golden Helix analysis were used. DNA samples from four commercially available cell lines were also included for use in further functional follow-up studies to assess the copy number states in these cell lines. NanoString's workflow has three different primers built into the array to obtain triplicate results for further confirmation.

2.5. RNA qPCR for EYA2 and WNK1 Protein Expression:

We performed quantitative-RT-PCR (qRT-PCR) to test the gene expression among samples with different copy number states to find a relationship between the copy number state and the expression. Total RNA was first extracted from cell lines and reverse transcribed. The cDNA was then used for TaqMan qPCR analysis. Each experiment was repeated three times, using three replicates of the same sample in each run to ensure consistency of results.

SYBR® Green I (Life Technologies, CA) was used to perform real-time PCR with synthesised single-stranded cDNA from RNA extracted from Ov Ca cell lines (A2780, HOSE-636 and OV CAR3& CAOV3). RNA expression analysis was performed for the selected genes (*WNK1* and *EYA2*) to identify their expression amount and to link the expression to the copy number state of each sample. The coordinates of CNV segments that overlapped the genes of interest were detected by Golden Helix software analysis (*WNK1*: coordinates hg19, chr12:867,000-875,750; coordinates hg18, chr12:737,261-746,011 and *EYA2* coordinates hg19, chr20:45,778,000-5,788,000; coordinates hg18, chr20:45,211,407-45,221,407). According to these coordinates, primers were designed to detect the mRNA of the following:

*WNK1*_5′ CAGTTGCGACACAACCCTCGGT

*WNK1*_3′ TGGGAAGCCCTGGTACAGAACA

*EYA2*_5′ AGACCTGGCTACAGCTCCGAGC

*EYA2*_3′ ACATTGACACAGTTGGGCCGGGA

The output of the qPCR was analysed to detect the mRNA expression of every sample. The results were compared to the CNV state of the DNA of the same sample for the same segment previously tested by Golden Helix analysis and confirmed by TaqMan qPCR.

2.6. Data Analysis:

The data were analysed with Affymetrix GTC software to generate copy number, LOH and segmentation data from the raw intensity values (CEL files). Analysis was performed on the detected CNV regions using the following statistical tests for CNV association analysis: Correlation/Trend Test, Armitage Trend Test, Exact Form of Armitage Test, (Pearson) Chi-Squared Test, Fisher's Exact Test, Odds Ratio with Confidence Limits, Analysis of Deviance, F-Test, Logistic Regression and Linear Regression.

3. Results

On the one hand, for each histopathological type (irrespective of the stage), some CNVs were shared by all subtypes of Ov Ca. On the other

hand, other CNVs specific to each subtype were not identified when the samples were compared to HapMap. In another trial to find CNVs among different groups according to the stage of cancer (among cases of stages I, II, III and IV), all shared some CNVs, but when comparing each stage separately to HapMap, unique CNVs (correlated with higher grade and stage) could be found among each stage, which may be of prognostic importance in Ov Ca. In this study, most cases were of the serous carcinoma subtype (31.1%), followed by endometrioid carcinoma (20.5%), clear cell carcinoma (12.9%) and other types (34.4%). Similarities in gene expression could be seen between the serous and endometrioid subtypes and between the clear cell and endometrioid subtypes.

Association analysis was performed on the detected CNV regions, dividing the cases into two groups (Ov Ca samples and normal control group) using the statistical tests mentioned in section 2.6. For association analysis, the –log10 p-value for all chromosomes was plotted for all cases using a Manhattan Plot of the corrected data. Association analysis detected six significant top hits that characterised cancer cases from the control ethnically homogeneous age-matched normal group (see Table 1). The most significant detected CNVs are at six locations: 20q13.12 overlapping the *EYA2* gene, 12p13.33 overlapping the *WNK1*, 12p11.22 overlapping the *KLHDC5*, 13q13.3 overlapping the *CSNK1A1L*, 18q22.1 overlapping the *CCDC102B* and 5q11.2 overlapping the *PLK2* (see Figures 1 and 2). *KLHDC5*, *CSNK1A1L* and *CCDC102B* are pseudogenes that do not have previous research on their biological roles. The most significant detected genes that have p-values <0.05 are *WNK1* and *EYA2*, which have previously been described as the carcinogenesis pathways of many cancers: CN_623069 overlapping the *EYA2* (20q13.12) gene at location chr12:867,000-875,750 and CN_874978 overlapping the *WNK1* (12p13.33) gene at location chr20:45,778,000-45,788,000.

For validation studies using cell lines, the work focused on *WNK1* and *EYA2* genes and carried out DNA copy number analysis in conjunction with RNA expression analysis. DNA copy number analysis used two methods, namely, TaqMan qPCR and NanoString assays. The Ov Ca cell lines included SKOV3, OVCAR5, 2008, CAOV3, A2780 and HOSE636. DNA was also extracted from HapMap cell lines NA12878 and NA10851, which were used as controls.

The first validation method, TaqMan qPCR, was performed to measure the copy number states of the DNA extracted from different Ov Ca samples and the HapMap cell lines that were previously analysed using Golden Helix. The second technique for validation was NanoString, and the results of the two validation steps were compared with each other and with the arrays detected in CN states.

The two assays for validation correctly recapitulated the results of the arrays in the Golden Helix analysis for HapMap cell lines. The CN states for ovarian cancer cell lines were also in agreement with the predominant CN state in ovarian cancer detected by Golden Helix arrays analysis. Therefore, the copy number states of the most significant CNVs associated with Ov Ca were confirmed and validated.

Table 1 shows the results of our extensive association analysis using Golden Helix software with the p-value for each association. The most significant hits that overlap genes are presented in descending order of p- values.

The output data of the association analysis, which nominated significant CNV regions, distinguish ovarian cancer from normal cases. These CNVs overlap known genes and pseudogenes listed in the associated gene column.

The first column in the table represents the chromosome, the second column is the cytoband location in the chromosome and the third column is the exact starting genomic coordinate of the CNV segment in the chromosome. The p-value is presented in the highlighted fifth column, and the last column lists the –log10 p-value.

Table 1: The output data of association analysis using Golden Helix software nominated significant CNV regions that distinguish ovarian cancer from normal cases

Chromosome	Cytoband	Position	Associated gene	T-test P	Regression-log10 P
20	q13.12	45780094	EYA2	0.002857	2.61044
12	p13.33	869251	WNK1	0.010271	2.052746
12	p11.22	28095546	KLHDC5	0.01287	1.963284
13	q13.3	38072024	CSNK1A1L	0.040633	1.43374
18	q22.1	66742743	CCDC102B	0.045041	1.385111
5	q11.2	57326015	PLK2	0.052274	1.312454

Figure 1: Heat map of the copy number gain overlapping the *EYA2* gene in cancer cases

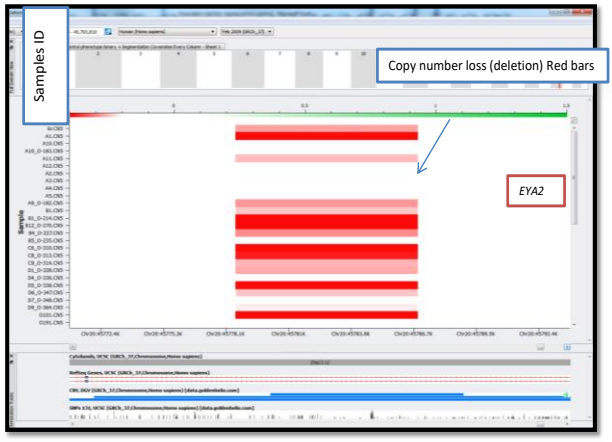

Figure 1 is a heat map of the copy number loss detected at the location of the *EYA2* gene (Chr20q13.12) in normal cases; however, ovarian cancer cases show a copy neutral (CN2) state in the same segment. This means that cancer cases have more copies in the Chr20q13.12 location than the normal control group. Red bars represent the areas of copy number loss, while the white bars represent CN2 cases. The X-axis represents the exact position of the copy number variation segment, and the Y-axis represents the ID of the studied ovarian cancer samples.

Figure 2: Heat map of the copy number gain overlapping the *WNK1* gene in cancer cases

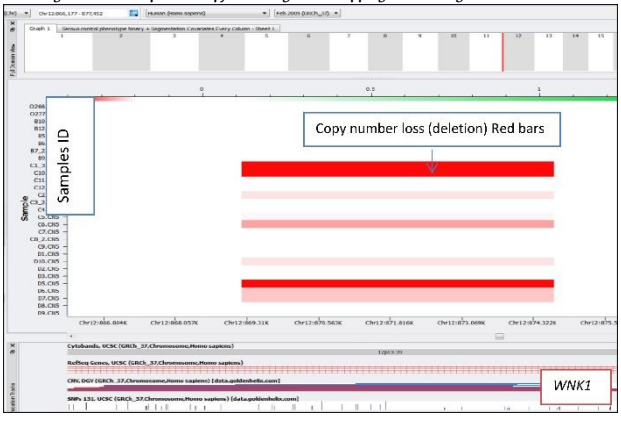

Figure 2 is a heat map of the copy number loss detected at the location of the *WNK1* gene (Chr12p13.33) in normal cases. The ovarian cancer cases show either a CN2 state or copy number gain (CN3) in the same segment. This means that cancer cases have more copies in the Chr12p13.33 location than the normal control group. The red bars represent the areas of copy number loss, while the white bars represent the CN2 regions. The X-axis represents the exact position of the copy number variation segment, and the Y-axis represents the ID of the studied ovarian cancer samples.

In the CNVs overlapping *EYA2* and *WNK1*, deletion is more frequent in normal and in some low-grade cancers, while Ov Ca is chiefly CN2 or has CN3, giving a clue to the possible oncogenic role of these two genes in Ov Ca.

Validation of the resulting top hits was performed using two different techniques: TaqMan qPCR and NanoString copy number analysis. The results of the two validation steps were compared to each other and the arrays detected in CN states. TaqMan qPCR was performed to measure the copy number states of the DNA extracted from different Ov Ca and the HapMap cell lines that were previously analysed using Golden Helix. The CNV segment that overlaps the *EYA2* gene (see Figure 3) shows that the copy number states used in NanoString (panel-A) are identical to the results obtained from Golden Helix analysis (panel-B). This means that the CNV segment overlapping the *EYA2* gene was successfully validated and its true CNV is related to Ov Ca. For the CNV segment that overlaps the *WNK1* gene (see Figure 4), the copy number states used in NanoString (panel-A) are identical to the results obtained from Golden Helix analysis (panel-B). The CNV segment overlapping the *WNK1* gene was successfully validated and its true CNV is related to the cancer cases.

Figure 3: Expected versus detected CNV (*EYA2*)

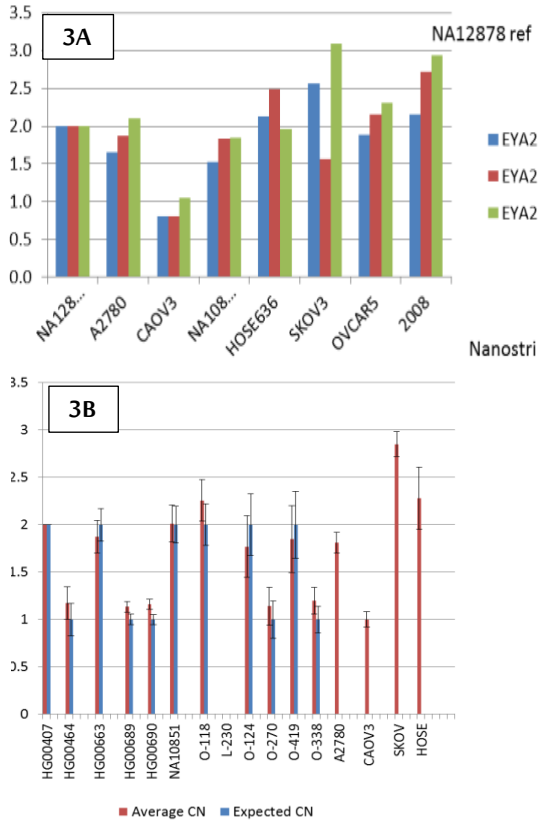

Figure 3 presents illustrations of copy number state validation using the NanoString technique. Picture (A) shows the copy number state of different samples using three different primers. Picture (B) compares the CN state of NanoString and the CN state of TaqMan qPCR with successful validation. The CNV segment that overlaps the *EYA2* gene is tested, and all samples of the NanoString copy number states (Figure 3A) are identical to the results obtained from the Golden Helix analysis in Figure 3B. This means that the CNV segment overlapping the *EYA2* gene is successfully validated and its true CNV is related to the carcinoma samples.

Figure 4. Expected versus detected CNV (*WNK1*)

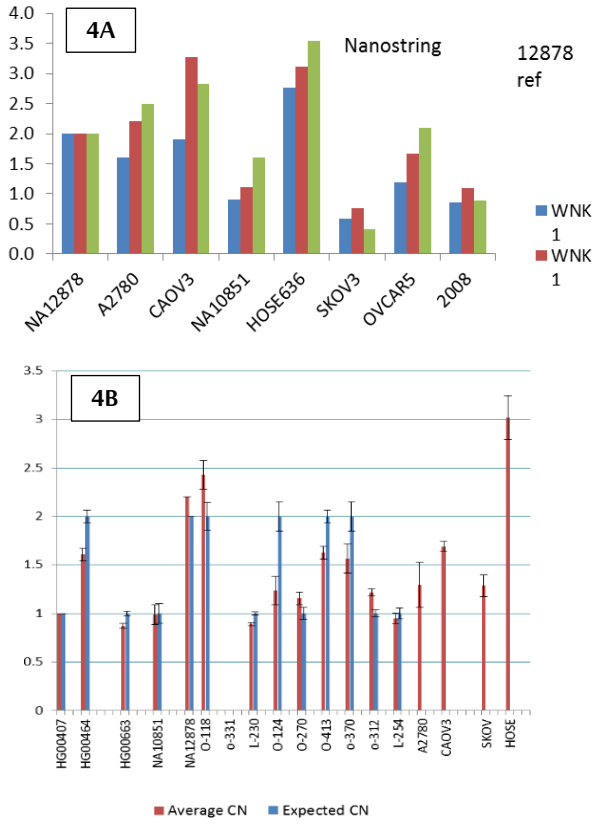

Figure 4 presents illustrations of copy number state validation using the NanoString technique. Figure 4A shows the copy number state of different samples using three different primers. Figure 4B compares the CN state of NanoString and the CN state of TaqMan qPCR with successful validation. The CNV segment that overlaps the *WNK1* gene is tested, and all samples used in the NanoString copy number states (Figure 4A) are identical to the results obtained from the Golden Helix analysis in Figure 4B. This means that the CNV segment overlapping the *WNK1* gene is successfully validated and its true CNV is related to the serous carcinoma samples.

The two assays for validation correctly recapitulated the results of the Golden Helix arrays analysis for the HapMap cell lines. The CN states for Ov Ca cell lines were also in agreement with the predominant CN state in ovarian cancer detected by Golden Helix arrays analysis. Therefore, the copy number states of the most significant CNVs associated with Ov Ca were confirmed and validated.

For *WNK1*, the RNA expression was seen to increase with a higher DNA copy number in the same segment (Figure 5). However, in the case of *EYA2*, no consistent correlation was detected between the DNA copy number state and the RNA expression level in the cell lines tested (Figure 6), which may be explained by the presence of many factors affecting the *EYA2* expression other than the copy number state. Our study confirmed frequent amplification at the segment overlapped by *WNK1* (Chr12p13.33), with concordant mRNA overexpression in many Ov Ca cell lines.

Figure 5. The DNA copy number state and mRNA expression of the *WNK1* gene

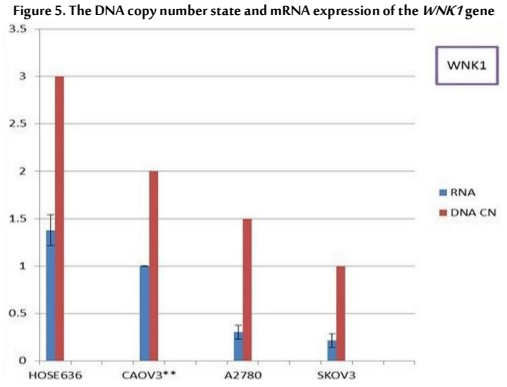

Figure 5 shows the validation of Ov Ca samples with different copy number states detected by Golden Helix analysis using TaqMan qPCR. The graph compares the DNA copy number state (red bars) and the RNA expression state of the same samples (blue bars).

Figure 6. The DNA copy number state and mRNA expression of the *EYA2* gene

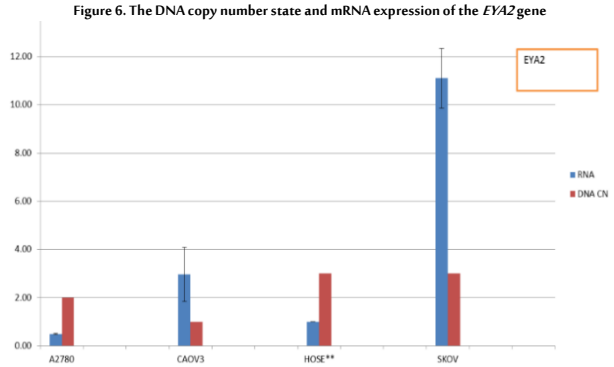

Figure 6 shows the validation of Ov Ca samples with different copy number states detected by Golden Helix analysis using TaqMan qPCR. The graph compares the DNA copy number state (red bars) and the RNA expression state of the same samples (blue bars).

4. Discussion

The high mortality rate of Ov Ca arises from a lack of diagnostic symptoms, the absence of effective biomarkers to identify early stages and missing reliable prognostic markers. The heterogeneous nature of Ov Ca histologic subtypes may affect treatment modalities and disease prognosis (Kobel *et al.*, 2008). Evaluating the molecular changes, copy, content and structure associated with epithelial Ov Ca arising in distinct genetic backgrounds offer approaches to therapeutic intervention (George *et al.*, 2013).

In this study, a germline association study using blood from patients with ovarian cancer was carried out using the genome-wide human SNP array 6.0, which contains 906,600 probes for SNPs and 946,000 probes for the detection of CNV loci. Affymetrix GTC was used to generate copy numbers, LOH and segmentation of the raw data (CEL files). The presence of CNVs specific to each histopathological subtype in this study indicates that each subtype of ovarian cancer has distinctive CNVs that may lead to better identification of the pathogenesis of Ov Ca to invent early screening tools. Therefore, future analysis integrating chromatin features into the CNV selection process could identify other CNVs missed in this analysis that are associated with cancer risk (Walker *et al.*, 2017). Ov Ca heterogeneity will also have affected the ability to identify risk loci, as common variant risk regions are different for different histopathological types of Ov Ca (Lawrenson *et al.*, 2019). In this study, a germline association study using blood from patients with ovarian carcinoma using the genome-wide human SNP array 6.0 can reveal novel genes worthy of follow-up for cancer susceptibility.

The most significant CNVs detected were 20q13.12 overlapping the *EYA2* gene and 12p13.33 overlapping the *WNK1* gene (p-values <0.05), which have previously been described as some of the carcinogenesis pathways of many cancers. The *EYA* family form a mutual transcription factor complex that supports the proliferation and survival of progenitor cells. The expression of *EYA* in adult tissue is associated with the origin and development of diverse tumour types. *EYA* proteins also contain protein tyrosine phosphatase activity, which plays a crucial role in breast cancer growth and metastasis as well as guiding cells to the repair pathway upon DNA damage (Blevins *et al.*, 2015). The epigenetic silencing of *EYA2* is a common incident in pancreatic cancers, and its constant expression limits the growth and metastases of adenocarcinoma (Vincent *et al.*, 2014). *EYA* proteins have multipurpose biochemical activities, which are associated with different cellular functions, and elevated *EYA* ranks of expression might enhance resistance to DNA-damaging therapeutic regimens frequently consumed in cancer therapy (Blevins *et al.*, 2015; Tadjuidje and Hegde, 2013). *EYA* proteins affect advanced cancer progression via their role as pro-proliferative and anti-apoptotic factors. A tough DNA damage response pathway comprises the regulation of H2AX phosphorylation via *EYA2* to facilitate proper cell cycle progression upon injury (Sousounis *et al.*, 2020). The most eminent cell cycle regulators, cyclin D1, p27 and *c-myc*, are target genes of the *EYA* transcriptional stimulators. CDK6 expression enhances the degradation of the *EYA2* protein (Kohrt *et al.*, 2014). Overexpression of micro RNAs-30a in lung adenocarcinoma cells can inhibit cell migration and invasion, which is partially attributed to the decrease in *EYA2* expression. These results may be used in the future as a new prospective target for the treatment of lung adenocarcinoma (Yuan *et al.*, 2016).

Using SNP arrays to detect CNVs has many benefits such as being cost-effective and requiring fewer samples per experiment compared to other techniques (Winchester *et al.*, 2009). For validation studies using cell lines focused on *WNK1* and *EYA2* genes, CN states for ovarian cancer cell lines are also in agreement with the predominant CN state in ovarian cancer detected by Golden Helix array analysis. If copy number-driven alteration in gene expression is a common attribute of the genomic panorama, it is likely that epigenetic modifications will further polish gene expression in favour of tumour growth (Bowtell, 2010).

Herein, the detected CNVs overlapping *EYA2* and *WNK1* give a clue to the possible oncogenic role of these two genes in Ov Ca. Amplification at these locations is associated with high-grade tumours, which have a worse overall survival rate. *WNK1* and *EYA2* amplifications are significantly associated with high-grade tumours, which indicates the possible oncogenic activity of both genes, and *WNK1* and *EYA2* may serve as prognostic markers for Ov Ca. These results are in agreement with other studies that suggest the oncogenic role of *WNK1* and *EYA2* in different cancer types (Costa *et al.*, 2015; Li *et al.*, 2018). A significant association is also found between higher expression and end-stage ovarian cancer along with poor prognosis (Xu *et al.*, 2019). *WNK1* plays an essential role in early embryonic angiogenesis regulation through oxidative stress response kinase. Emerging evidence suggests that ion homeostasis is important for cell migration in many cell types and Akt phosphorylation of *WNK1* may affect its targeting and interaction with downstream targets (Huang *et al.*, 2020). Generally, the functional diversity of *WNK1* offers positive feedback loops for the amplification of tumour growth. *WNK1* can promote tumorigenesis by stimulating tumour angiogenesis. Thus, a *WNK1* signalling cascade might be a multi-purpose focus for blended cancer therapy. The inhibition of *WNK1* may be a potent anti-cancer therapy (Sie *et al.*, 2020).

Targeting *EYA* may inhibit the growth and progression of multiple tumour types (Zhou *et al.*, 2018). *EYA2* has a higher expression and copy number in ovarian cancer than in normal human ovaries, which is consistent with similar observations in breast cancer that are in concordance with this work (Xu *et al.*, 2019). In the case of *EYA2*, no consistent correlation has been detected between DNA copy number state and RNA expression level in the cell lines tested. This may be attributed to any other biological factor that increases the *EYA2* expression that is not related to the increase in the copy number state. Further research is needed to detect the cause of the high expression of *EYA2*. A somatic study of ovarian cancers, especially epithelial, identified a high expression of *EYA2* mRNA in different types of malignant cells. A high copy number is directly associated with the mRNA expression level of *EYA2*. In Ov Ca cell lines, up-regulation of *EYA2* has also been shown to push the tumour growth of cells and is related to the genomic amplification of its locus in Ov Ca specimens. Up-regulation of *EYA2* has been associated with the activation of tumour growth and decreased overall survival, especially in epithelial Ov Ca (Zhang *et al.*, 2005), which aligns with these results. The 20q13.12 amplification was observed in multiple tumours, and it encodes 11 genes, one of which is *EYA2*. However, the current association contrasts with other findings that reported improved overall survival with chr20q13.12 amplification (Xie *et al.*, 2012). Early-stage Ov Ca is commonly asymptomatic, and nearly 75% of women have the progressive disease at diagnosis. Hence, Ov Ca is the leading cause of death among gynaecological cancers, as it represents 4% of all cancers in women. Overall survival is highly dependent on the stage of Ov Ca, and five-year survival is 80–90% in patients with early-stage compared with 25% for patients with advanced stage. Therefore, early screening for Ov Ca increases survival among patients (Alexandrova *et al.*, 2020).

Gene amplification and an increase in the DNA copy number of a chromosomal segment often lead to an increase in oncogene expression in many human cancers (Li *et al.*, 2017). There is frequent amplification at the segment overlapped by *WNK1* (Chr12p13.33) with concordant mRNA overexpression in many Ov Ca cell lines. Members of the *WNK* family have been shown to modulate MAPK signalling. *WNK1* activates the ERK5-MAP kinase (MAPK) cascade in addition to the regulatory role of the TGF-β-Smad signalling cascade (B. H. Lee *et al.*, 2007). Phosphatidylinositol 3-kinase PI3K-activating hormones phosphorylate *WNK1* by AKT1 and SGK1 (Cheng and Huang, 2011). Transient transfection studies of *WNK1* revealed that overexpression of *WNK1* leads to the activation of ERK5 (Wang and Tournier, 2006). Many studies emphasise that *WNK1* functions as a MAP4K in the MAPK pathways (Sun *et al.*, 2006). *EYA2* is a co-factor for the *SIX1* gene that induces TGF-β signalling to activate the epithelial-mesenchymal transition as well as stimulate metastasis (Blevins *et al.*, 2015). MAPK positively regulates *EYA*. EGFR/RAS/MAPK signalling phosphorylates *EYA* as a downstream, and the two MAPK phosphorylation sites detected in *EYA* positively standardise *EYA* activity in vivo (Tootle *et al.*, 2003). The activation of RAS/MAPK can potentiate *EYA*-mediated transactivation, whereby in the absence of RAS/MAPK signalling, it can trigger some transcription. However, when the signal occurs, this function is potentiated such that target genes may be activated to advanced levels. An alternative role for RAS/MAPK is to grant more activation potential to *EYA* to overwhelm the negative regulation of specific target genes (Silver *et al.*, 2003).

Our study may present comprehensive data assembled to our knowledge; however, there are potential limitations. The sample size is small, so it is necessary to apply the same study outline to a larger sample size to confirm our association analysis for ovarian cancer. Furthermore, although using peripheral blood samples and cell lines allows the analysis of the germline, tissue samples from the same patients could strengthen the results. The tissue samples in this study, however, had intrinsic noise, which led to unreliable results.

To summarise, preliminary evidence for CNVs in two potential oncogenes (*WNK1* and *EYA2*) using Affymetrix SNP 6.0 arrays was found and was associated significantly with high-grade Ov Ca cases. This may confer risk to Ov Ca that needs extended study on a bigger sample size to confirm the association analysis.

5. Conclusion

There is evidence of cross-talk between the *WNK1* and *EYA2* genes and the *MAPK* signalling pathways, which is a common ovarian carcinogenic pathway detected in many reports. The detected CNV genes might not be the aetiology, but they may overlap *WNK1* and *EYA2*, which share the same pathway. The potential diagnostic and therapeutic values of these genes may help in early diagnosis and proper management to minimise the high mortality rate of this disease. Ovarian carcinomas are associated with copy number gains at the segments of DNA overlapping *WNK1* and *EYA2*. This may support the hypothesis that both *WNK1* and *EYA2* have an oncogenic effect on ovaries. The copy number gain of the *WNK1* gene is associated with a higher expression of mRNA.

6. Recommendations

Applying the same study outline in a bigger sample size to confirm the association analysis of ovarian cancer is recommended. Performing functional analysis of *WNK1* and *EYA2* on ovarian cancer cell lines (overexpression and knocking down) will explore the effect of gene overexpression on cell proliferation or division and will knock down the same genes by comparing the effect on cells.

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