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Correlations between *c-myc* gene copy-number and clinicopathological parameters of ovarian tumours

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ARTICLE INFO

Article history:
Received 22 July 2005
Received in revised form
9 November 2005
Accepted 16 November 2005
Available online 3 February 2006

Keywords:
Ovarian cancer
c-myc amplification
Tissue microarrays

ABSTRACT

The objective of this study was to investigate increases in *c-myc* gene copy-number in ovarian tumours, and to analyze their correlations with clinicopathological parameters. Here we applied FISH on TMA (tissue microarrays) containing 507 ovarian tumour samples from different malignancy, histology, stage and grade. Overall, we found high frequency for *c-myc* copy-number increases (38.5%) in ovarian cancers: 22.1% amplifications and 16.4% gains. We established *c-myc* amplification in more than 30% in endometrioid and mixed epithelial ovarian carcinomas. *c-myc* gains were found in a high proportion (42.9%) of clear cell carcinomas. We found associations between *c-myc* copy-number changes and clinicopathological parameters of ovarian tumours such as degree of malignancy and histological type. We suggested that *c-myc* amplifications are characteristics for endometrioid, and *c-myc* gains for clear cell ovarian cancers. We suggest that copy-number increases of *c-myc* and 20q13.2 represent a possible mechanism for the regulation of the pathway *STK15* – *c-myc* – hTERT.

1. Introduction

Ovarian cancer, although second in incidence as a gynaecologic cancer, causes more deaths than all other gynaecologic cancers combined. This tumour type represents 4% of all cancer cases. It is tied with pancreatic cancer as the fourth most common cause of cancer death in women, being preceded by cancer of the lung, breast and colon and rectum.[1] Unfortunately, the pathogenesis of this disease is poorly understood, but a deeper knowledge of the biology of ovarian cancer would be the base for the development of new therapeutic concepts. The understanding of the molecular pathogenesis of ovarian cancer has been hindered by the lack of sufficient numbers of specimens at early stage disease because of its frequent diagnosis at an advanced stage.

As in many other cancers, the initiation and progression of this cancer involve accumulation of genetic changes, such as rearrangements, amplifications, and deletions affecting critical genes for cell growth, differentiation and death.[2] Ovarian carcinomas show complex cytogenetic rearrangements.[3] Bayani and colleagues investigated a set of ovarian cancers and identified by spectral karyotyping, that chromosomes 3, 8, 11, 17 and 21 had the highest frequencies of structural and numerical aberrations.[4] The most frequently affected chromosomal regions in ovarian tumours, detected by comparative genomic hybridization (CGH), are 3q, 8q and 20q, often with high-level amplification.[5] Genes, activated by such chromosomal alterations may be primary mediators of the clonal progression of cancer. Comparative genomic hybridization has determined the copy-number increases of the region

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0959-8049/$ - see front matter
doi:10.1016/j.ejca.2005.11.022

8q24 as very common in ovarian tumours (35–76%).[6–15] This region contains the gene *c-myc* for transcription factor, which was found to cause the formation of murine ovarian tumours that were similar to human ovarian carcinomas.[16]

c-myc protein binds to the promoters of at least five genes. It is a transcription activator of proliferation factors, and also an inhibitor of factors suppressing growth.[17] *c-myc* induces the transcription of the genes *e2f1*, *e2f2* и *e2f3* and blocks the function of p53 by binding the promoter of *p21*.[18] Finally, there is evidence for direct activation of telomerase activity by c-myc, and is known to be a key activator of human telomerase reverse transcriptase (hTERT) transcription.[19] Moreover, c-myc appears to be mediator of telomerase activation by Aurora-A kinase gene (*STK15*), the key gene of 20q13.2 amplicon, as the later simultaneously induces c-myc expression and telomerase activity in a dose-dependent manner.[20] *c-myc* is targeted in vitro as anti-oncogene therapy by specific oligonucleotides.[21,22] This pinpoints *c-myc* as critical cancer-emerging gene.

Tissue microarray (TMA) technology enables us to perform molecular analysis in large numbers of tumour samples, leading to discoveries that underpin the clinicopathological significance of gene copy-number alterations. Here, we have applied fluorescent in situ hybridization (FISH) of *c-myc* on TMA, containing large number of ovarian tumours from different malignancy, histology, tumour stage and histological grade in order to establish the frequency of *c-myc* copy-number alterations in different ovarian tumours, to evaluate the genetic heterogeneity of ovarian tumours, to analyze the correlations of *c-myc* copy-number changes with clinicopathological parameters, and to compare the *c-myc* copy-number changes with other related gene copy-number alterations.

2. Materials and methods

2.1. Ovarian tumours tissue microarray (TMA)

TMA containing 503 ovarian tumour samples from formalin-fixed paraffin-embedded blocks of 507 patients was constructed. The blocks were collected from the gynaecopathological department of University Hospital of Obstetrics and Gynaecology, Sofia. There were 234 malignant, 24 low malignant potential and 245 benign tumours. Ovarian tumours vary considerably in their histological type. TMA contained specimens from all histological variants: 222 serous, 67 mucinous, 72 endometrioid, 8 clear cell, 5 Brenner, 25 mixed epithelial, 26 non-differentiated, 21 non-classified, 25 granulose-cell, 36 germline-cell tumours (teratomas, dysgerminomas). Histological heterogeneity may be associated with genetic heterogeneity. The haematoxillin-eosin (H&E) stained slides from all tumours were reviewed prior to construction by a single pathologist and representative areas of each tumour were determined. Tumour stage and grade were defined according to FIGO and WHO criteria.[23]

Sections of the microarray provide targets for parallel in situ detection of DNA, RNA and protein targets in each specimen on the array, and consecutive sections allow the rapid analysis of hundreds of molecular markers in the same set of specimens. For TMA construction, a hematoxilin and eosin (H&E)-stained section was made from each block to define representative tumour areas. Tissue cylinders with a diameter of 0.6 mm were punched from tumour areas and brought into a recipient paraffin block using a custom-made precision instrument.[24] Samples were distributed in one regular-sized recipient paraffin block containing 507 specimens. Five micrometer sections of the blocks were transferred to glass slides using a paraffin-sectioning aid system (adhesive coated slides, adhesive tape, UV-lamp; Instrumedics Inc., Hackensack, NJ).

2.2. Fluorescent in situ hybridization (FISH)

Fluorescent in situ hybridization is a rapid method, highly specific and sensitive for evaluation of particular genetic aberration. It represents direct visualization of fluorescence labeled DNA sequence on interphase or metaphase nuclei. Here we used FISH for detection of copy-number of *c-myc*. Prior to hybridization, the slides were treated with Paraffin Pretreatment Reagent Kit (Vysis). FISH was performed using a locus-specific probe for *c-myc* labeled in Sectrum Orange (Vysis, Cat #30-190006). Denaturation of the DNA was carried out at 75 °C for 10 min (probe mixture) or 5 min (slides). The probe mixture was applied to the slides and hybridized overnight in a moist chamber at 37 °C. The post-hybridization washes were performed as described in "LSI procedure" (Vysis). Slides were counterstained with DAPI in anti-fade. The presence of >4 copies per cell or tight clusters in at least 10% of tumour cells was considered as amplification according to the instruction of Vysis (Pathvysion). Presence of more than two but $\leqslant$4 gene signals in at least 10% of tumour cells was considered a "gain".

2.3. Statistical analysis

The relationship between copy-number changes and clinicopathological data was estimated using χ^2 test and *P*-value was calculated. $P < 0.05$ was required for significance.

3. Results

3.1. c-myc copy-number changes

A TMA including 507 ovarian tumour samples was analyzed by FISH for *c-myc* copy-number changes (amplifications and gains). FISH was successful in 75% of the tumours (380 samples – 280 malignant, 23 low malignant potency and 77 benign tumours). Copy-number changes of *c-myc* were found in 38.5% of all ovarian malignancies, in 26.1% of tumours with low malignant potential and in 7.8% of benign ovarian tumours (Table 1). These alterations were associated with the degree of malignancy ($P < 0.0001$).

3.2. c-myc amplifications and malignancy

c-myc amplification was strongly associated with the degree of malignancy of ovarian tumours, the frequency of this alteration increased statistically from low malignant potential (8.7%) to malignant (22.1%) tumours and was 0 in benign adenomas ($P < 0.0001$) (Table 1).

Table 1 – *c-myc* copy-number changes in malignant, low malignant potency and benign ovarian tumours

Degree of malignancy	Total	N	Copy-number changes		
			Total	G	A
		n (%)	*n* (%)	*n* (%)	*n* (%)
Malignant	280	172 (61.4%)	108 (38.5%)	46 (16.4%)	62 (22.1%)
Low malignant potential	23	17 (73.9%)	6 (26.1%)	4 (17.4%)	2 (8.7%)
Benign	77	71 (92.2%)	6 (7.8%)	6 (7.8%)	0
P-value			$P < 0.0001$	n.s.	$P < 0.0001$

N, normal copy-number; *G*, gains; *A*, amplifications; *n*, number of cases.

3.3. *c-myc amplifications and histological type*

The highest frequency of *c-myc* amplification was revealed in mixed epithelial (40.9%) and endometrioid carcinomas (30.8%), followed by mucinous (26.9%), serous and non-classified (22.2%), undifferentiated (9.4%) epithelial malignant tumours and non-epithelial malignant tumours (11.1%). *c-myc* amplification was absent in clear cell carcinomas, but was found in 8.3% of serous and in 12.5% of mucinous low malignant potency tumours (Table 2).

3.4. *c-myc amplifications and ovarian tumour phenotype (stage and grade)*

The relationship between *c-myc* amplifications and tumour phenotype is summarized in Table 3 regarding tumor grade and in Table 4 regarding tumor stage. There was no statistically significant difference between frequencies of *c-myc* amplification in different grade tumours (G1 – 21.1%, G2 – 27%, G3 – 18.3%) and tumour stage: stage I – 20.8%, stage II – 21.2%, stage III – 23.8% (Table 4).

We have analyzed the occurrence of *c-myc* amplification in stages I, II and III for each histological group. The data show that *c-myc* amplification is significantly associated with advanced tumour stage in endometrioid ovarian carcinomas ($P < 0.05$).

The analysis of *c-myc* amplification frequency in different grade tumours from each tumour stage (three tumour grade in three tumour stages) in every histologic type did not show any correlation.

Table 2 – *c-myc* copy-number changes in different histological groups

Histological type	N	G	A	Total
	n (%)	*n* (%)	*n* (%)	
Malignant tumours				
Serous	88 (57.5%)	31 (20.3%)	34 (22.2%)	153
Mucinous	18 (69.2%)	1 (3.8%)	7 (26.9%)	26
Endometrioid	7 (53.8%)	2 (15.4%)	4 (30.8%)	13
Mixed	12 (54.5%)	1 (4.5%)	9 (40.9%)	22
Clear cell	4 (57.1%)	3 (42.9%)	0	7
Undifferentiated	22 (68.7%)	7 (21.9%)	3 (9.4%)	32
Unclassified	13 (72.2%)	1 (5.6%)	4 (22.2%)	18
Non-epithelial	8 (88.9%)	0	1 (11.1%)	9
Low malignant potency				
Serous	8 (66.7%)	4 (30.8%)	1 (8.3%)	13
Mucinous	7 (87.5%)	0	1 (12.5%)	8
Endometrioid	1	0	0	1
Mixed	1	0	0	1
Benign tumours				
Serous	19 (90.5%)	2 (9.5%)	0	21
Mucinous	8	0	0	8
Endometrioid	27 (93.1%)	2 (6.9%)	0	29
Brenner	1	1	0	2
Mixed	2	1	0	3
Unclassified	3	0	0	3
Non-epithelial	11	0	0	11

N, normal copy-number; *G*, gains; *A*, amplifications; *n*, number of cases.

Table 3 – *c-myc* copy-number changes in different grade tumours

Grade	N	G	A	Total
	n (%)	*n* (%)	*n* (%)	*n*
G1	47 (61.8%)	13 (17.1%)	16 (21.1%)	76
G2	56 (56%)	17 (17.0%)	27 (27%)	100
G3	69 (66.3%)	16 (15.4%)	19 (18.3%)	104
P-value		n.s.	n.s.	280

N, normal copy-number; *G*, gains; *A*, amplifications; *n*, number of cases.

3.5. *c-myc gains*

There was no statistically significant difference in the frequencies of *c-myc* gains in malignant tumours (16.4%), tumours with low malignant potential (17.4%) and benign ovarian tumours (7.8%) (Table 1).

3.6. *c-myc gains and histological type*

The frequency of *c-myc* gain was highest in clear cell carcinomas (42.9%) in contrast to *c-myc* amplification, followed by undifferentiated (21.9%), serous (20.3%), endometroid (15.4%), non-classified (5.6%), mixed (4.5%) and mucinous carcinomas (3.8%) (Table 2). Unlike *c-myc* amplification, *c-myc* gains were not found in the small group of non-epithelial tumours.

In tumours with low malignant potential, *c-myc* gain was revealed in 30.8% of serous tumours and was not found in other histological types. Among the benign ovarian tumours, *c-myc* gain was present in 9.5% of serous, 6.9% of endometrioid, 1 mixed and 1 Brenner tumours (the number of the last tumours was very low).

Table 4 – c-myc copy-number changes in ovarian cancers of different stages

Tumour stage	N n (%)	G n (%)	A n (%)	Total n
Stage I	83 (66.4%)	16 (12.8%)	26 (20.8%)	125
Stage II	19 (57.6%)	7 (21.2%)	7 (21.2%)	33
Stage III	70 (57.4%)	23 (18.8%)	29 (23.8%)	122
P-value		n.s.	n.s.	280

N, normal copy-number; G, gains; A, amplifications; n, number of cases.

Table 5 – Correlation between c-myc and 20q13.2 copy-number increases

Copy-number	Normal 20q13.2 n (%)	20q13.2 copy-number increases n (%)	Total
Normal c-myc	56 (72.7)	11 (37.9)	67
c-myc copy-number increases	21 (27.3)*	18 (62.1)*	39
Total	77	29	106

*P < 0.001.

3.7. c-myc gains and ovarian tumour phenotype (stage and grade)

c-myc gains were observed in 17.1% of G1, 17% of G2 and 15.4% of G3 tumours without statistical significance of this alteration in different grade tumours (Table 3).

Concerning tumour stage, c-myc gains tended to be more frequent in stage II–III not reaching statistical significance (Table 4).

We have analyzed the occurrence of c-myc gain in stages I, II and III for each histological group. Gains of c-myc did not show correlation with tumour stage in any histological group. We analyzed c-myc gain frequency in different grade tumours from each tumour stage (three tumour grade in three tumour stages) in every histologic type as well, and did not find any correlation.

3.8. c-myc and 20q13.2 copy-number increases

As c-myc is up-regulated by Aurora-kinase gene (the key gene in 20q13.2 amplicon), we compared the copy-number changes of c-myc and 20q13.2[25] in 106 ovarian tumours (Table 5). We found that 62.1% of 20q13.2 copy-number increases were combined with c-myc copy-number increases, whereas there was only 27.3% c-myc gains among tumours with normal copy-number for 20q13.2 (P < 0.001).

4. Discussion

In this study, we applied FISH on TMA for an investigation of c-myc copy-number changes in a large number of ovarian tumours from different malignancy, histology, stage and grade. The region 8q24, containing the gene, has been reported as gained in many CGH studies of ovarian tumours.[5–15] There is limited number of FISH analyses for c-myc alterations.[26]

Overall, we found high frequency for c-myc copy-number increases, 38.5% in ovarian cancers: 22.1% amplifications and 16.4% gains (which are related to structural or numerical chromosomal changes). The finding was similar to the results of some of the CGH studies,[9,12,14] although many of them reported higher frequency for 8q gains (50–76%).[5–8,10,11,13,15] This discrepancy is linked to the limited resolution of the CGH and probably to the presence of other potential oncogenes in 8q. In a study of c-myc amplification by polymerase chain reaction in archival human ovarian carcinomas, increased c-myc copy-number was found in 17% of the tumours when a control from the same chromosome was used, but in 37% of cases if control from another chromosome was used.[27] Using Southern hybridization analysis, five of seventeen (29.4%) tumour samples demonstrated amplification of c-myc oncogene and 54.5% of cases in another study.[28,29] The differences may be the result of subjective interpretation. The most reliable method is FISH and our results for c-myc copy-number increases in ovarian cancers (38.5%) were similar to the results (40%) of Wang and colleagues, who utilized the same method for the analysis of 40 ovarian tumours.[26]

For the first time, we have analyzed by FISH the occurrence of c-myc copy-number changes in large number of ovarian tumours from different malignancy (benign, low malignant potency and malignant). We have discovered that c-myc amplification was strongly associated with malignancy (P < 0.0001), whereas c-myc gains did not. Our investigation by the reliable method of FISH showed the presence of c-myc copy-number increases in low malignant potency and in small proportion of benign ovarian tumours. Using different methods (CGH and immunohistochemistry), other authors have reported that increased copy-number alterations of chromosome 8 play an important role in the development and differentiation of serous low malignant potency tumours (it occurred in 38% of them),[14] the most common genomic imbalance in this tumour group were gains of 8 or 8q,[7,30] while the over-expression of c-myc was present in 37.3% of all ovarian tumour tissues and in 63.5% of serous adenocarcinoma tissues.[31] Probably, c-myc gene and protein alterations appear at the early stages of ovarian tumour genesis.

The TMA technology enabled us to analyze the occurrence of c-myc copy-number changes in ovarian tumours of different histology types. We defined that endometrioid and mixed epithelial ovarian carcinomas were affected in more than 30% by c-myc amplification, whereas clear cell carcinomas had c-myc gains in high proportion (42.9%). Interestingly none of the last type tumours demonstrated c-myc amplification. This confirmed that c-myc gain and amplification are different molecular events. Using immunohistochemical analysis, Plisiecka-Halasa and colleagues revealed a high incidence of c-myc overexpression in endometrioid and clear cell carcinomas (70%), and suggested its role in the development of these tumour types.[32] Copy-number alterations of 8q, analyzed by CGH, were also found more frequently in clear cell[8] and in ovarian cancers arising in endometriosis.[33] Our current data suggest that c-myc amplifications are characteristic of endometrioid, and c-myc gains for clear cell ovarian cancers.

We have analyzed the relationship of *c-myc* copy-number alterations with tumour stage and grade. We performed this analysis in all tumours and in each histological type. Overall, there was no statistically significant association of *c-myc* amplification or gain with tumour stage or grade. However, in the group of endometrioid carcinomas, *c-myc* amplification was associated with advanced tumour stage ($P < 0.05$). Data in the literature about prognostic significance of *c-myc* copy-number alterations are controversial. In a CGH study 8q24 gain was associated with high-grade and showed tendency for prevalence in advanced stage.[5] In another study this alteration was linked to platinum resistance[34] in contrast to another.[28] Suehiro and colleagues found association of 8q24 gains with disease-free duration.[8] According to other authors, the survival curve for those patients who have *c-myc* amplification appears to show a trend toward poorer survival.[26] Our data do not suggest a role of *c-myc* copy-number alterations for stage progression of primary ovarian cancer except for endometrioid cancer. However, *c-myc* copy-number increases are common findings in this tumour type.

Finally, we compared the occurrence of *c-myc* copy-number alterations with those of 20q13.2, analyzed in the same tumour.[25] Our results show that a high proportion (62.1%) of 20q13.2 copy-number increases was associated with *c-myc* copy-number increases also. Yang and colleagues suggested that the key gene of 20q13.2 amplicon *STK15* activates human telomerase reverse transcriptase (hTERT) by targeting the *c-myc* transcription factor.[20] We suggest that copy-numbers of the two genes are very often increased together, and is probably one of the mechanisms for the regulation of this pathway.

In conclusion, the high number of ovarian tumours analyzed in our TMA, allowed us to rapidly identify associations between *c-myc* copy-number changes and clinicopathological parameters of ovarian tumours, such as degree of malignancy, and histological type. The data provide a picture on the incidence of *c-myc* gains and amplifications in benign, low malignant potential and malignant ovarian tumours as well as relationship between *c-myc* gains and amplifications and tumour stage and grade. We have also established an association between copy-number changes of *c-myc* and 20q13.2, which suggested a possible mechanism for the activation of the *STK15-c-myc-hTERT* pathway.

Conflict of interest statement

None declared.

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