Tissue Microarray Analysis of HER2 copy number changes in Ovarian cancer

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Abstract

This study was accomplished in Medical University – Pathologoanatomic Department of the Clinic of Thoracic Surgery. Sofia,. Bulgarian, were collected the paraffin blocks, by using new technology Tissue microarrays (TMA) and Fluorescence In Situ Hybridization (FISH)) on different type of ovarian tumors . The method of FISH applied on TMA with HER1 and HER2 specific probes proved itself as the most commonly used and valuable analysis for routine HER2 status detection and copy number changes . The results showed in out of all 603 ovarian carcinomas examined, the number of successfully hybridized for HER2 oncogene is 303 (50.2%) 192 of them are malignancies tumors(179 epithelial and 13 non epithelial) ,18 low malignant ovarian tumors(just epithelial), 93 – Benign ovarian tumors(77 epithelial and 16 non epithelial) . We established HER2 amplification in malignancies tumors only 20 (10.41%) ,19 (10.61%) epithelial and in 1 (7.69%) non epithelial) . There is no amplification in tumors with low malignant potential and Benign ovarian tumors. While we found HER2 gains in malignancies tumors , low malignant ovarian tumors and Benign ovarian tumors only 19 , malignancies 14 about (7.29%) ,low malignant 2 (11.11%) and in Benign ovarian tumors 3 (3.22%). Epithelial malignant tumors among the highest incidence of HER2 amplification was detected in Clear cell carcinomas 2 (28.57%) and rank them Endometrioid 2 (16.66%), Serous 9 (10.46%), undifferentiated tumors 2 (11.11%) and mixed(combined) 2 (11.76%), Mucinous 1 (6.25%) and Unclassification 1 (4.55%). HER2 amplification was found in 1 of 13 non epithelial malignant tumors, Sex-cord stromal tumours 1 (7.69%). HER2 gains in malignancies tumors was detected in serous 5 (5.81%) ,endometrioid 1 (8.33%) , undifferentiated 2 (11.11%) , mixed (combined) 2 (11.7%) and mucinous1 (6.25%), in low malignant ovarian tumors was detected HER2 gain only in mucinous 2 (11.11%) and Benign ovarian tumors only in serous 3 (7.69%).Were preserved at the grouping of tumor samples according to the WHO classification.

1. Introduction

Ovarian cancer is the most common fatal cancer of the female. At the time of writing, it is the fourth most common cause of cancer death in women in the U.K., after breast, lung, and colorectal cancer (Jemal et al,2007). Approximately estimated new cases 21,550 and deaths 14,600 from ovarian cancer in the United States in 2009 (Ohsaki et al., 2000). The histological classification of ovarian cancer is complex, with a large number of histological subtypes. According to WHO, types of ovarian cancers in women age 20 are as follows: 1-Surface epithelial-stromal tumour, also known as ovarian epithelial carcinoma, is the most common type of ovarian cancer. It includes serous tumour, endometrioid tumor and mucinous cystadenocarcinoma. 2- Sex cord-stromal tumor, including estrogen-producing granulosa cell tumor and virilizing Sertoli-Leydig cell tumor or arrhenoblastoma, accounts for 8% of ovarian cancers. 3-Germ cell tumor accounts for approximately 30% of ovarian tumors but only 5% of ovarian cancers. 4- Mixed tumors, containing elements of more than one of the above classes of tumor histology. During the last years, several new agents targeting specific and critical pathways for ovarian cancer(Olayioye , 2001). One of these

agents HER2/neu (also known as ErbB-2, *ERBB2*) stands for "Human Epidermal growth factor Receptor 2" and is a protein giving higher aggressiveness in ovarian cancer(Santin et al,2008).

HER2-neu is member of the erbB gene family and encode for transmembrane receptor-type tyrosine-protein kinases (Olayioye , 2001). It is normally involved in the signal transduction pathways leading to cell growth and differentiation in ovarian cancer (Santin et al,2008, Coussens,1985). Overexpression of HER-2/neu, is seen in approximately 5% to 20% of ovarian cancers. HER2 can be determined by several methods. The most commonly used methods are Fluorescence in situ hybridization (Wolff et al,2007), which detects gene amplification by measuring the number of copies of the HER2 gene in the nuclei of tumor cells(Sliwkowski et al,2004) *HER2* amplification and overexpression are established through several molecular-biological and biochemical techniques – Southern blotting, Western blotting, PCR, ELISA. The immunohistochemical (IHC) examination of *HER2* receptor protein is another commonly used method for *HER2* status detection(Rubin et al,1993), and it is measures the number of HER2 receptors on the cell surface and therefore detects receptor overexpression (Sliwkowski et al,2004) .

2- Materials and methods

The tumor samples in this study were obtained from morphologically representative paraffin-embedded tumor tissues a total of 603 ovarian carcinomas fixed in paraffin blocks were examined. The first step was to create a database of patients' data including the year of disease diagnosis, passport data, histological diagnosis, disease stage, number of each sample and clinical course information. Then, the paraffin blocks, according to their numbers in the database, were collected. The paraffin blocks, arranged by number and year of obtainment, were preserved at the Pathologoanatomic Department of the Clinic of Thoracic Surgery. A grouping of tumor samples according to the WHO classification and their histology followed.

Two tissue microarrays, each including three tumor sample replicas were constructed. Prior to microarray construction, all tumor sections were examined by a pathologist. The histological type of the examined tumors varied significantly, for which the tissue array contains samples of almost all histologic versions.

The material for this study has been completely submitted by the Medical University – Sofia, Clinic of Thoracic Surger.

Methods

All these procedure were applied according to the thesis of (Al-Zeyade, 2008) .

Fluorescence *In Situ* Hybridization (FISH).

The method of Fluorescence *In Situ* Hybridization (FISH) applied on adequately prepared ovarian tumor tissue microchips (tissue microarrays, TMA) was used for *HER2* gene examination. As for DNA is a stable structure, paraffin-embedded tissues make possible its detection. For this reason, tumors are conserved in donor paraffin blocks and recipient paraffin block-derived sections are used for the genetic analysis. The method of FISH applied on TMA with *HER2* specific probes proved itself as the most commonly used and valuable analysis for routine *HER2* status detection.

Tissue microarrays technology

Screening of ovarian tumor clinical stage, histological type and clinical course is performed using the method of TMA. The role of examined *HER1* and *HER2* oncogenes as well as ovarian tumor recurrence and progression could be thus objectified. Using this method, the diagnostic, prognostic and therapeutic significance

of each candidate gene participating in ovarian tumor occurrence and development could be determined. The knowledge of these yet unclear tumors could be further deepened by constructing an ovarian tumor tissue microarray for expression analysis using FISH with *HER1* or *HER2* probes, which, on its turn, could benefit the use of these markers for selecting the precise therapy and most adequate therapeutical approach. According to this technology, tissue specimens are represented as formalin-fixed or freshly frozen and cool ethanol-fixed tumors subsequently embedded in donor paraffin blocks, thus preserving DNA and RNA. Recipient block-embedded tumor specimens could be derived from various organs, in this case – from ovaries. Taken from previously selected tumor specimen areas in the donor paraffin blocks and are subsequently transferred to a recipient block by using a specifically designed device, histologically representative miniature cylinders are. Using a microtome, the recipient block is dissected and the derived sections are mounted on slides. The technique enables the obtainment of about 1000 tumor specimen samples from different donor paraffin blocks, their arrangement on a single recipient block and further transfer as a section on a single slide, thus forming a single microchip (microarray). Finally, the slide with the tumor specimen sample is ready for further processing – different *in situ* analyses.

Recipient block construction

1. For recipient block constructing, only these donor paraffin blocks are selected, for which the pathologoanatomic register has found to include only tumor specimen.
2. After the required number of donor blocks has been collected, a precise examination of embedded tumor specimen for selecting the most appropriate site of its extraction is performed. The procedure is as follows:

   Using a microtome, a donor paraffin block-derived thin section is obtained and mounted on a slide;Hematoxylin and eosin stain is performed.Under a microscope, the microtome section is examined for selecting the appropriate, histologically representative tumor cellular site. The selection of the donor block site is determined in correspondence to the stained section on the slide.The selected tumor cellular site is contoured on the sample and is used as a marker for relevant specimen acquisition from each donor paraffin block.The slide is removed immediately before block surface puncture.

3. Using the specifically designed device, whose principle scheme is presented on (Al-Zeyade, 2008) the constructing of tissue microarrays is performed.The steel needle penetrates into the recipient block and forms the recipient bed by a sudden vertical manual movement.A stand is placed over the prepared recipient block, protecting it from damage and serving at the same time as a base, on which the donor paraffin block is placed.A manual replacement of the first vertical needle by the second one, along the XY axes, is performed.The selected representative tumor cellular area of the donor paraffin block is positioned under the second hollow needle.The needle penetrates into the donor paraffin block by a careful vertical manual movement and gains the selected specimen, forming a miniature cylinder with a 0.6mm diameter and 6mm height.The donor block and the underlying stand are removed and the recipient block is revealed with the prepared recipient bed on it.Then, the containing in the needle tumor specimen (tumor tissue core) is pushed out in the 45-20mm recipient bed on the recipient paraffin block .The samples from all donor paraffin blocks are subsequently placed in one recipient paraffin block as each section is mounted on a separate slide thus constructing a microarray . Thus, a certain gene might be examined simultaneously in 1000 tumor tissue samples . The tissue

microarrays technology enables the examination of tumor tissue morphology, protein expression and active genes .

Recipient block organization

The recipient paraffin block is constructed on the base of a pre-created scheme. Each recipient block is marked with specific label (Hauptmann et al,2002), which make each tumor has its code comprising the recipient block number. For example, 1A7b is the place of a certain tumor probe and means: first block, first quadrant, seventh row, second probe(Hauptmann et al,2002, Al-Zeyade, 2008). The distance between two adjacent probes is of about 800 μm (0.8 mm). Thus, one 45-20 mm recipient block may contain approximately 1000 tumor cylinders. The data for the tumors on the microarray are kept in a special file including tumor's code (localization in the microarray), histologic type, stage and degree,patient's name, age,survival and therapeutic response, etc.

Obtainment of recipient block sections

Before starting block dissection, the block surface should be leveled and smoothed. For achieving this, the recipient block is placed in a moist chamber at 37ºC for 10-15 min. Using a clear slide, the block surface is pressed so it could become even. By this step the number of obtained slides per block is increased. Using a microtome, the recipient block is dissected into 5-8 μm thick 100-200 sections. The sections are mounted on adhesively covered slides. An adhesive band and an UV lamp enabling tide fixation of microarrays to the slides are required. Using a microtome with a specifically designed adhesive is quite a useful method of recipient block dissection. FISH analysis is performed on the prepared slides for assessing *HER2* gene amplification. Two subsequent hybridizations for the examined gene.

Method of fluorescence *in situ* hybridization (FISH)

Applied in ovarian tumors, the method of FISH enables the localization of a specific fluorescent labelled DNA sequence directly on metaphase chromosomes or in interphase nuclei of the examined tumor cells. The used dual-colour FISH contains a combined FISH-probe consisting of a locus DNA-probe labelled in red – for the examined by us gene (*HER2*, 17q) and a centromeric DNA-probe labelled in green – for the respective chromosomes (17-a). Thus, the specific chromosome regions of the genes examined in our study and the centromeres of their chromosomes are detected by counting their copy numbers.

On preparation for examining, the specimen is embedded in paraffin blocks. Thus, interphase nuclei (containing the DNA examined) or metaphase chromosomes obtained from lymphocyte cultures are fixed on slides. The examined double-stranded DNA is denatured and after heating in a formamide solution is turned into single-stranded. Denaturation is applied also to DNA probe (sonde). Sondes for analyzing centromeric or telomeric regions are suitable for numerical aberrations. The subsequent *in situ* hybridization is performed through DNA sonde dripping on the examined DNA on the slide. DNA sondes hybridize with the entire chromosome or a part of it. They may be directly labelled with fluorochrome or indirectly with modified desoxinucleotids conjugated with haptene (*biotin* or *digoxigenin*). The slide is washed with formamide – SSC, to eliminate the non-conjugated DNA sonde. Finally, a detection of the hybridized sonde is performed. In case of indirect *biotin* labelling, the detection is achieved by *avidin* conjugated with the green fluorochrome *FITC* or the red *rhodamine*. In case of indirect *digoxigenin* labeling, the detection is achieved by *digoxigenin-antibodies* conjugated with fluorochrome *FITC* or *rhodamine*. Nuclei and chromosomes are stained in blue by the fluorochrome *DAPI*.

Specimen treatment procedure

1. Slides preparation

Before hybridization, the slide undergoes preparation aiming at proteolysis of nucleic membrane proteins. Thus, subsequent penetration of the DNA probe into cellular nuclei on the slide is enabled.

Procedure steps: The slide is placed in a cuvette with xylol 3x for 10 min. (this step is performed in an oven) – purpose: deparaffinization of tumor specimen.The slide is placed in an incubator 2x for 5 min. in 96% ethanol (ethanol is prepared immediately before procedure) – purpose: dehydration, fixation and further preparation of tumor specimen.The slide is placed on *in situ* PCR apparatus at 40°C (the PCR apparatus is preprogrammed) – purpose: drying of tumor specimen. The slide is placed in a preheated cuvette with *pretreatment solution* (Al-Zeyade, 2008), in a water bath at 80°C for 15 min.The slide is placed in a cuvette with distilled water 2x for 1 min. – purpose: washing of tumor specimen.The slide is placed in a preheated cuvette with *protease buffer* (Al-Zeyade, 2008), in a water bath at 37°C for 30 min. – purpose: protease activated protein proteolysis. This step is preceded by protease addition to the protease solution. 1 ml preheated protease solution is pre-added to the dried enzyme, the latter is well dissolved and the mixture is added to the cuvette with protease solution, pre-mixed additionally with the slide when it is placed. On microarray preparation, this step is of duration of about 30 min. – the time needed by the enzyme to decompose completely the specimen's proteins, the labeled DNA to penetrate maximally in them and to hybridize with the tumor cellular nuclei. In isolated paraffin non-embedded probes, the slide is examined under a phase-contrast microscope and the time for pepsin effect is determined by examined cell morphgology. The slide is placed for a second time in a cuvette with distilled water 2x for 1 min. – purpose: washing of tumor specimen.The slide is placed for 5 min. in each ethanol series: 70%, 85% and 96% (ethanol is prepared immediately before procedure) – purpose: dehydration of tumor specimen.The slide is let air dry completely at room temperature.

2. DNA probe preparation

The DNA probes used in our study are submitted with the courtesy of the Institute of Pathology in Basel, Switzerland. The FISH probes used for genetic analysis of *HER2* copy numbers in ovarian tissue microarrays are locus-specific dual-colour probes representing a dual sequence labelled mixture – *Spectrum green*-labelled for the centromeric region of 17-a chromosome and *Spectrum orange*-labelled for examined gene (*HER2*): LSI HER2 Spectrum orange /CEP 17 Spectrum green: Using an automatic pipettor, certain quantities of the already prepared DNA probe (earlier centrifuged for better sedimentation), hybridization buffer and redistilled water are mixed in a test-tube. The final probe volume depends on the number of regions for hybridization. Each region is dripped with a 10 μl DNA probe (Al-Zeyade, 2008).The mixture, called "mix" is placed on vortex and is centrifuged for a second time for 2-3 sec. – purpose: better probe homogenization before its dripping on the slide for hybridization.

3. DNA probe denaturation: A 10 μl DNA probe (ready mix) is transferred in eppendorf. The probe is denatured in dark, at 75°C on PCR apparatus (which is preprogrammed on TMA 73TO37 programme) for 10 min. The DNA probe is directly labelled with fluorochrome through modified labelled nucleotide insertion.The 10 μl labelled probe (the probe's quantity is determined by the tissue microarray number) is immediately dripped on the dried tissue microarray sample on the slide.A coverslip is placed and cemented – purpose: successful hybridization process.The sample and the probe are placed at 73°C on PCR apparatus (on the same TMA 73TO37 programme

through which the temperature is gradually decreased to 37°C) – purpose: tumor's DNA denaturation and probe's DNA re-denaturation. On this step, placing the sample in dark is of importance, since the DNA probe is fluorescently labeled. After temperature's decrease to 37°C, the slide is removed.

4. *In situ* hybridization of the samples:The obtained sample is placed in a moist hybridization chamber and is let at 37°C for at least 16 hours (overnight) in an thermostat – purpose: complementary conjugation of sample's and tumor cell DNA sequences;

5. Washing of non-specifically conjugated DNA probes:

The coverslip is removed from the slide.The sample is placed in a water bath at 72°C in a preheated cuvette with wash buffer (Al-Zeyade, 2008) for 2 min. in dark – purpose: elimination of non-specifically conjugated DNA probes.The sample is transferred in a cuvette with wash buffer (Al-Zeyade, 2008) at a room temperature for 2 min. in dark. The sample is washed for 5 min. with distilled water in dark – purpose: washing of SSC solution.The sample is let to air dry at a room temperature in dark – purpose: prevention of fluorochrome labelled probe's decomposition under light.

6. Contra-staining:

- 12 μl (0.1 μg/ml) fluorescent DNA dye – *DAPI/antifade* (Al-Zeyade, 2008), is dripped on the already dried sample – purpose: visualization in blue (*DAPI*) of separate cellular nuclei by which the fluorescent signals from isolated cells could be assessed;
- The sample is covered with a 24x32 mm or 24x24 mm coverslip;
- Until its examining under a microscope, the sample is storaged at -20°C.

Sample's microscope examination and analysis

A fluorescent microscope "Olympos BX60" for DNA probe detection is used. The microscope is connected to *MetaSystems*, *ISIS-C* computed system for fluorescent image recording through CCD imaging. The analysis is based on counting the signals for the gene and respective centromeric chromosome region in the cells using the appropriate filter. Normal diploid cells have dual signals corresponding to two homologous chromosomes. The presence of one or more than two signals in centromeric probes is assessed as numerical aberration. The presence of one gene signal and two centromeric is accepted as deletion. The presence of one centromeric signal is assessed as monosomy. The slide is carefully examined. For each tissue cylinder, a maximum number of cells is analyzed, as the signals for the gene and centromere are compared. The green signal corresponds to the centromeric region of the examined chromosome (17-a), and the red signal corresponds to the examined gene (*HER2*). Normally, the number of signals for the gene and centromere is equal to two (red signal: green signal = 2 : 2). Gene oncogene amplification is assessed in case of at least 3-fold increase of gene signals compared to centromeric for the same chromosome. Genetic gain (additional genetic material i.e. increased gene copy number) is assessed in case of at least 1.5-fold (but less than 3-fold) gene signals compared to centromeric. The results for each tumor are represented in tissue microarray's scheme which we were showing above and are placed in a pre-created file including tumor probe availability in the recipient block. exact XY coordinates and the whole information available for individual tumors.

Results and Discussion :

Out of all 603 ovarian carcinomas examined, the number of successfully hybridized for HER2 oncogene is 303 (50.2%) , 192 of them are malignancies

tumors (179 epithelial and 13 non epithelial) ,18 low malignant ovarian tumors(just epithelial), 93 – Benign ovarian tumors(77 epithelial and 16 non epithelial) . The availability of unsuccessfully hybridized tumors is due to various reasons: 1) loss of material; 2) an insufficient number of cells; 3) a lack of signal after hybridization; 4) irrelevant specimen acquisition from the donor block during tissue microchip preparation. We established HER2 amplification in malignancies tumors only 20 (10.41%) ,19 (10.61%) epithelial and in 1 (7.69%) non epithelial) . Not found amplification in tumors with low malignant potential & Benign ovarian tumors. While we established HER2 gains in malignancies tumors , low malignant ovarian tumors and Benign ovarian tumors only 19 , malignancies 14 about (7.29%) ,low malignant 2 (11.11%) and in Benign ovarian tumors 3 (3.22%) tables (4,5,6).

Table 4. Frequency of HER2 copy number in ovarian carcinomas with different histological type in malignant tumors

Histological Type Malignant Tumors		Normal of HER2 signals	HER2 genetic gain	HER2 amplification	TOTAL
Epithelial	N	149	11	19	179
	%	83.24	6.14	10.61	
Serous	N	72	5	9	86
	%	83.72	5.81	10.46	
Mucinous	N	14	1	1	16
	%	87.5	6.25	6.25	

Endometrioid	N	9	1	2	12
	%	75	8.33	16.66	
Clear cell	N	5	0	2	7
	%	71.42		28.57	
Brenner tumors	N	1	0	0	1
	%	100			
Combined	N	13	2	2	17
	%	76.47	11.76	11.76	
Undifferentiated tumors	N	14	2	2	18
	%	77.77	11.11	11.11	
Unclassification	N	21	0	1	22
	%	95.45		4.55	
Non epithelial	N	9	3	1	13
	%	69.23	23.07	7.69	
Sex-cord stromal tumours	N	3	0	1	4
	%	75		25	
Granulosa cell tumours	N	2	0	0	2
	%	100.0			
Embryonal carcinoma	N	4	3	0	7
	%	57.14	42.85		
TOTAL	N	158	14	20	192
	%	82.29	7.29	10.41	

Table 5. Frequency of HER2 copy number in ovarian carcinomas with different Histological Type in Low Malignant Tumors

Low Malignant Tumors		**Normal of HER2 signals**	**HER2 genetic gain**	**TOTAL**
Epithelial	**N**	**16**	**2**	**18**
	%	**88.88**	**11.11**	
Serous	**N**	**8**	**0**	**8**
	%	**100.0**		
Mucinous	**N**	**5**	**2**	**7**
	%	**71.42**	**28.57**	

Endometrioid	**N**	**1**	**0**	**1**
	%	**100.0**		
Clear cell	**N**	**0**	**0**	**0**
	%			
Brenner tumors	**N**	**0**	**0**	**0**
	%			
Combined	**N**	**2**	**0**	**2**
	%	**100.0**		
Undifferentiated tumors	**N**	**0**	**0**	**0**
	%			
Unclassification	**N**	**0**	**0**	**0**
	%			
Non epithelial	**N**	**0**	**0**	0
	%			
Sex-cord stromal tumours	**N**	**0**	**0**	**0**
	%			
Granulosa cell tumours	**N**	**0**	**0**	**0**
	%			
Embryonal carcinoma	**N**	**0**	**0**	**0**
	%			
TOTAL	**N**	**16**	**2**	**18**
	%	**88.88**	**11.11**	

Table 6. Frequency of HER2 copy number in ovarian carcinomas with different histological type in benign tumors

Benign Tumors		**Normal of HER2 signals**	**HER2 genetic gain**	**TOTAL**
Epithelial	**N**	**74**	**3**	**77**
	%	**96.10**	**3.81**	
Serous	**N**	**36**	**3**	**39**
	%	**92.3**	**7.69**	
Mucinous	**N**	**12**	**0**	**12**
	%	**100.0**		

Endometrioid	**N**	**19**	**0**	**19**
	%	**100.0**		
Clear cell	**N**	**0**	**0**	**0**
	%			
Brenner tumors	**N**	**2**	**0**	**2**
	%	**100.0**		
Combined	**N**	**5**	**0**	**5**
	%	**100.0**		
Undifferentiated tumors	**N**	**0**	**0**	**0**
	%			
Unclassification	**N**	**0**	**0**	**0**
	%			
Non epithelial	**N**	**16**	**0**	**16**
	%	**100.0**		
Sex-cord stromal tumours	**N**	**2**	**0**	**2**
	%	**100.0**		
Granulosa cell tumours	**N**	**2**	**0**	**2**
	%	**100.0**		
Embryonal carcinoma	**N**	**12**	**0**	**12**
	%	**100.0**		
TOTAL	**N**	**90**	**3**	**93**
	%	**96.77**	**3.22**	

DISCUSSION

Up to now, the most studies on frequency of *HER2* alterations in ovarian tumors examined gene overexpression in a small number of tumors (Meden et al,1997, Khalifa et al,1997). Various CGH examinations have found an additional gene material in 17q (Kraggerud et al,2000). The analysis of deferent type of ovarian carcinoma has shown on frequency of HER2 alterations in deferent chromosomes and deferent frequency of chromosome areas(Watanabe et al,2001). *HER2* amplification and overexpression are established through several molecular-biological and biochemical techniques. (Kraggerud et al,2000, Watanabe et al,2001)

Our study has used the FISH method for assessing the alterations in *HER2* copy number. The highly informative technology of tissue microarray has enabled the simultaneous analysis of a great number of ovarian tumors. Thus, the FISH method applied on TMA with specific probe for the examined *HER2* oncogene, has realized amplification and genetic gain frequency detection, thus enabling evaluation of the input of this oncogene rearrangements for the ovarian tumor occurrence and progression. HER2 protein was overexpressed in all tumors with gene amplification, implicating gene amplification as one mechanism for overexpression according to the results of Reinmuth et al (Resta et al, 1993) using double-differential polymerase chain reaction technology. Furthermore, the correlation between increasing levels of HER2 protein expression and increased gene copy number suggests that the additive effect of gene copies is an important mechanism for HER2 protein expression (Hirsch et al, 2002). Studies of HER2 gene amplification in other tumours have reported lower incidences of gene amplification relative to receptor overexpression, implying that in most tumours gene amplification is not the prevailing mechanism of HER2 overexpression, and that it is also unlikely to be of diagnostic value for most solid tumours. (Rolitsky et al,1999)The underlying mechanisms of HER2 protein

overexpression are not understood completely. Gene amplification was proposed as a possible mechanism for many years, but recent studies indicate that other mechanisms may play a important role such as post-translational changes or the length of polymorphisms in intron 1 of the EGFR gene(Kersting et al,2004) .

Aberrant *HER2* copy numbers (gains and amplifications) were found in 17.70% of malignant ovarian tumors (epithelial and non-epithelial). Using the same method, Rolitsky *et al* (1999)Their evaluation of archive tumor material by FISH, however, has been carried out in a limited number of tumors (Werness et al, 1999), mainly of endometriod type. Ross *et al*. (1999) performed another study using FISH for detecting of *HER2* gene amplification. They have established even a higher frequency of *HER2* amplification (66%) in 40 of a total of 61 ovarian epithelial carcinomas. Bian et al. demonstrated amplification in 9 of 65 adenocarcinomas (14%) (Watanabe et al,2001). The reasons for the differences in various study results may lay in the limited number of tumors analyzed as well as in differences of amplification definitions. Our collection included ovarian tumors of all histologic types and subtypes. These various histologic types of ovarian tumors have demonstrated difference in *HER2* gain and amplification frequencies. We established *HER2* amplification associated with light cell carcinomas. This is in consent with Rolitsky et al., who have found that amplification is associated with serous subtypes (p=0.002), while *HER2* protein overexpression – with only light cellular subtype (Rolitsky et al,1999). Our results suggest that alterations in *HER2* copy number are particularly characteristic for the light cell ovarian tumors. Our suggestion is supported by the immunohistochemical (IHC) examinations of Fujimura et al., who have established the highest *HER2* protein overexpression in light cellular (42.9%) subtype (Ross et al,1999). Our study has not found amplification in tumors with low malignant potential. This is in consent with Ross et al (1999), who reported that *HER2* amplification is of exceptional occurrence in low malignant ovarian tumors compared to malignant ones (Kraggerud et al, 2000). Another study has found significantly decreased expression of *HER2* protein in low malignant potential tumors compared to carcinomas (Rolitsky et al,1999). The authors have suggested that the number of HER2 positive adenocarcinomas increases with disease progression.

Benign ovarian tumors show almost a lack of genetic gain (except for the serous subtype) and amplification, which means that *HER2* alterations are not an early phenomenon in ovarian tumorogenesis (Kraggerud et al,2000).This is supported by the study of Werness *et al.*, suggesting that increased HER2 expression occurs later in invasive tumor development and has its role in the late pathogenesis of ovarian carcinoma (Ross et al,1999). Finally, the great number of ovarian tumors analyzed by FISH on TMA enabled us to assess the frequency of *HER2* gene alterations, the gene being of great importance to cancerogenesis, and to examine it in the contest of various tumor phenotypes.

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استعمال تقنية المصفوفات الصغيرة للأنسجة TMA لتحليل تغييرات نسخ الجين HER2 في سرطان المبيض

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الخلاصة :-

أجريت هذه لدراسة في قسم الأمراض الوراثية السريرية لجراحة الصدر في الجامعة الطبية في صوفيا \ بلغاريا ، حيث جمعت عينات الأورام السرطانية لسرطان المبيض من المختبر المركزي الخاص لجمع وطمر العينات هنالك،

وباستخدام تقنيات حديثة مثل تقنية المصفوفات الصغيرة للأنسجة Tissue Microarrays TMA وتقنية التهجين داخل الخلوي بواسطة الفلورسين Fluorescence In Situ Hybridization FISH حيث أن استخدام طريقة TAM / FISH وباستعمال المجسات الخاصة للجين HER2 أثبتت بأنها من أكثر الطرق شيوعا وأهمية في تحليل ودراسة هذا الجين وتحديد حالاته وتغييرات أعداد نسخه. لقد أظهرت النتائج من بين 603 عينة لمختلف أنواع سرطان المبيض أن عدد العينات التي حصل لها تهجين لمجسات HER2 (HER2 c probes) بنجاح هي 303 عينة (50.25%) ، 192 منها كانت هي من النوع الورم الخبيث (179 نوع الأورام الطلائية و 13 الأورام غير الطلائية) ، 18 حالة من النوع الورم الأقل خبثا (اقل درجة من الورم الخبيث) وكانت جميعها من النوع الأورام الطلائية فقط ، و 93 كانت من نوع الورم الحميد لسرطان المبيض (77طلائي ، 16 غير طلائي) . وقد اتضح أن عملية تضخيم الجين HER2 حدثت فقط في بعض أنواع الأورام الخبيثة من أنواع سرطان المبيض المختلفة ولم يحصل تضخيم في الأورام الأقل خبثا وكذلك لم يحصل في الأورام الحميدة أيضا . إن هذا التضخيم ظهر في 20 حالة من أنواع الأورام الخبيثة أي حوالي (10.41%) ، 19 (10.61 %) من النوع الطلائية ، و1 (7.69%) غير الطلائية . بينما حصلت زيادة في جميع هذه الأنواع (الورم الخبيث والورم المتوسط والورم الحميد) حيث لوحظ ظهور ربح جيني (genetic gain) للجين HER2 في 19 حالة من جميع الأنواع الرئيسية الثلاث أي حوالي (6.27%) حيث في النوع الورم الخبيث 14 حالة حوالي (7.29%) وفي الورم المتوسط 2 حالة حوالي (11.11%) وفي الورم الحميد 3 حالة حوالي (3.22%) .

إن أورام المبيض الطلائية الخبيثة هي أكثر واهم الأنواع التي يحصل عندها تضخيم مناسب لجين HER2حيث كان التضخيم في النوع Clear cell carcinomas (28.57%) يليه Endometrioid (16.66%) ثم serous (10.46%) و Undifferentiated tumors (11.11%) و Mixed (11.76%) و النوع Mucinous ظهر بنسبة (6.25%) والنوع Unclassification بنسبة (4.55%) .
أما تضخيم ألجين HER2 في الأنواع غير الطلائية الخبيثة (Non epithelial malignant tumors) فقد وجد في نوع واحد من بين 13 نوع من الأورام غير الطلائية وهو النوع Sex-cord stromal tumours ونسبته (7.69%) . بينما أنواع أورام المبيض التي حصلت عندها ربح (زيادة) (gains) في الورم الخبيث ، فهي كل من serous (5.81%)، و Endometrioid (8.33%)، والنوع Undifferentiated tumors (11.11%)، والنوع mixed (combined) (11.7%)، والنوع Mucinous (6.25) أما في حالة سرطان المبيض المتوسط (الأقل حدة) فقد ظهرت زيادة في النوع Mucinous فقط وبنسبة (% 11.11) بينما في سرطان المبيض الحميد ظهر في النوع الطلائي Serous وبنسبة (7.69%) .
لقد تم تصنيف وحفظ عينات المجاميع هذه بموجب التقسيم العالمي لمنظم الصحة العالمية WHO.