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HER-2 and INT-2 Amplification Estimated by Quantitative PCR in Paraffin-embedded Ovarian Cancer Tissue Samples

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Competitive polymerase chain reaction (PCR) systems were developed for rapid and quantitative estimation of HER-2 (c-*erb*B-2) and INT-2 oncogene amplification in paraffin-embedded ovarian cancer tissue samples. The β-globin gene was used as reference and DNA from paraffin-embedded placenta tissue as single copy control. Reliability of the PCR method could be demonstrated by comparing dot blot data with PCR data of identical tumour samples. The PCR method was used to determine HER-2 and INT-2 copy numbers in 196 ovarian cancer samples. HER-2 and INT-2 were found to be amplified in 40 and 19%, respectively. In 8% HER-2 copy numbers were greater than five, but no high INT-2 copies were noted. Kaplan–Meier estimates did not reveal significant association with overall survival. Indirect correlation between HER-2 and INT-2 amplification was observed. The present PCR system is a valuable method for prospective and retrospective studies.
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INTRODUCTION

IN THE past few years oncogenes have been found to play significant roles in human tumorigenesis. The mode of oncogene activation was reported to vary in different types of tumours, as well as in different species. It was suggested that oncogene alterations were prognostic indices for the survival of patients [1–3]. The HER-2 oncogene, also known as c-*erb*B-2, is of interest because it is amplified in 10–30% of human mammary carcinomas and seems to be associated with overall survival and disease-free survival time [2, 4, 5]. Although the situation is still controversial [2, 6, 7], it is thought to be a valuable prognostic parameter [3, 8, 9] with worse prognosis for patients with amplified HER-2. The INT-2 oncogene, being a member of the fibroblast growth factor gene family, is thought to possibly play an important role in the genesis of solid tumours. It was found to be amplified in 4–23% of human mammary carcinomas [6, 10]. HER-2, *myc* and INT-2 genes are the most frequently amplified oncogenes in breast cancer [10, 11]. Two studies revealed a negative correlation between HER-2 and INT-2 copy numbers [6, 12].

Amplification data are usually obtained by quantitative Southern blotting or dot blotting which need considerable large sample volumes and are time consuming due to many experimental steps, scrupulous purification of the DNA and long autoradiography exposure times [13]. Moreover, degraded DNA is a source of error and results in wrong data. The use of a quantitative PCR system has a lot of advantages: the samples need not be purified extensively and even the presence of high fractions of degraded DNA (e.g. in paraffin-embedded tissue) does not interfere with the PCR reaction. In theory, one intact target molecule allows the synthesis of enough product for further investigation. Therefore, the sample volume for PCR-supported detection of gene amplification is some 100 times less than for blotting techniques. In addition, PCR experiments require only about a tenth of the time of Southern blotting. PCR techniques are fast, non-toxic and non-radioactive and they can be used to estimate amplification in large sample numbers and in short time. Therefore, this technique is useful for prospective as well as retrospective studies, in which the problem of degraded DNA arises.

The aim of the present study was to develop quantitative PCR systems for the estimation of HER-2 and INT-2 gene amplification in paraffin-embedded tissue samples of ovarian cancer, and to compare this data with results of classical blotting experiments.

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MATERIALS AND METHODS

DNA extraction

Tissue samples that were used for analysis were dissected free of non-tumorous material and examined by frozen section prior to paraffin embedding or deep freezing in liquid nitrogen.

For frozen tissue. DNA was extracted from frozen tissue sections of about 500 mg of ovarian cancer tissue by standard methods [13]. The DNA content of the obtained solution was estimated by measuring the OD_{260}.

For paraffin-embedded tissue. Two 10 μm paraffin-embedded sections of ovarian cancer tissue essentially free of stroma were deparaffinised with 1.5 ml isooctane. Each sample was vortexed thoroughly, incubated at 70°C for 5 min and spinned down. After four cycles of extraction the samples were dried in a SpeedVac concentrator and incubated with 65 μl proteinase K lysis buffer (1 mmol/l $CaCl_2$, 0.5% Tween 20, 10 mmol/l Tris–HCl pH 8.0 with 20 μg proteinase K) at 56°C for 4 h. The enzyme was denatured by boiling the samples for 20 min.

Two 10 μm thick tissue sections of each tissue sample contained too little DNA for exact determination of concentration. Therefore, the DNA concentration was estimated using the following formula:

$$\frac{\text{Volume of tissue section} \times \text{DNA content per diploid cell}}{\text{Average volume of cancer cell}}.$$

Areas of tissue sections were measured by a scale fixed to the object slide and were found to be 0.8 ± 0.2 cm^2. With a cancer cell having an average diameter of 25 μm, and a eucaryotic cell containing 13.3 pg of DNA [14], each DNA extraction should yield about 40 ng/μl DNA or 12 000 single copy targets/μl.

Dot blot experiments

The Amersham multiprime DNA labelling system was used for labelling the DNA probes with [α-^{32}P]-dCTP. The 1.6 kb *Eco*R1 fragment pHER2-436-2 was used as probe for the HER-2 oncogene [15] and a 0.9 kb *Sac*I fragment containing exon 2 served as probe for the INT-2 gene [16]. The β-actin gene served as reference gene in dot blot experiments and a 770 bp fragment of chicken actin obtained from Oncor was used as a probe. Dot blotting was carried out with 5 μg of DNA from frozen tissue on Oncor nylon membranes according to standard protocols [13]. Prehybridisation for 4 h and hybridisation overnight were performed in Hybrisol at 52°C. After washing the membrane at 52°C for 30 min with 2 × SSC containing 0.1% sodium dodecylsulphate (SDS) and two times for 1 h with 1 × SSC (0.15 mol/l NaCl, 0.015 tri-sodium citrate, pH 7.0) containing 0.1% SDS, it was sealed in a plastic bag and a Hyperfilm MP by Amersham was exposed for between 20 min and 2 days, depending on the activity of the dots, to ensure each signal was in the linear range of densitometry.

PCR experiments

In PCR experiments the β-globin gene served as reference and the primers PC03 (5'ACACAACTGTGTTCACTAGC3') and KM38 (5'TGGTCTCCTTAAACCTGTCTT3') yielding a 168 bp PCR product as published previously [17]. INT-2 primers (INT2a: 5'CAGAAGCAGAGCCCGGATAA3'; INT2b: 5'ACGCCAAGATGTCGCCAGGA3') were designed by a computer program [18]. These oligonucleotides were derived from the third exon of the INT-2 gene and yielded a 130 bp PCR product. HER-2 primers (HER2a: 5'CCTCTGACGTCCATCATCTC3'; HER2b: 5'ATCTTCTGCTGCCGTCGCTT3') were also computer designed and were derived from sequences flanking the transmembrane region. They resulted in a 98 bp PCR product. All primers were obtained from Biomedica, Vienna, Austria.

INT-2 system for DNA from frozen tissue. DNA (0.2 μg) was used in 100 μl reactions (0.20 mmol/l dNTP, 0.5 μmol/l of each primer: INT2a, INT2b, PC03, KM38, 2 U/100 μl Promega-Taq polymerase, 50 mmol/l KCl, 1 mmol/l $MgCl_2$, 10 mmol/l Tris–HCl pH 8.4, 0.001% gelatine, 0.1% Triton X100).

For DNA from paraffin-embedded tissue. HER-2 system: 1 μl of the DNA extraction supernatant was vortexed with 99 μl of reaction mix (0.25 mmol/l dNTP, 0.5 μmol/l of each primer: HER2a, HER2b, PC03, KM38, 2 U/100 μl USB-Taq polymerase, 100 mmol/l KCl, 3 mmol/l $MgCl_2$, 10 mmol/l Tris–HCl pH 8.4, 0.001% gelatine G2500).

INT-2 system: Three microlitres of the DNA extraction supernatant were vortexed with 97 μl of reaction mix (0.2 mmol/l dNTP, 0.5 μmol/l of each primer: INT2a, INT2b, PC03, KM38, 3 U/100 μl Promega-Taq polymerase, 50 mmol/l KCl, 5 mmol/l $MgCl_2$, 10 mmol/l Tris–HCl pH 8.4, 0.001% gelatine, 0.1% Triton X100).

In all different PCR systems the samples were incubated in a Thermocycler 60/2 for 30 cycles using the following parameters. Start cycle: 2 min at 94°C, 2 min at 55°C, 2 min 72°C; 30 cycles: 30 s at 94°C, 30 s at 55°C, 1 min 30 s at 72°C, time extension 1 s/cycle, extension last cycle 5 min.

In every PCR run a normal placenta DNA was used as single copy control, a highly amplified tumour sample as high copy control and another tube containing reaction mix without DNA as negative control.

There was no difference in product yield or specificity with buffers containing NaCl instead of KCl for the HER-2 and INT-2 PCR system, respectively.

Gel electrophoresis

The PCR products were separated by agarose gel electrophoresis using a 4% gel in 1× TAE (2% NusieveGTG, 2% low melting agarose by Biorad). The gels were stained with ethidium bromide in 1× TAE (2 mg/l). Each gel contained one lane with the single copy control (Fig. 1).

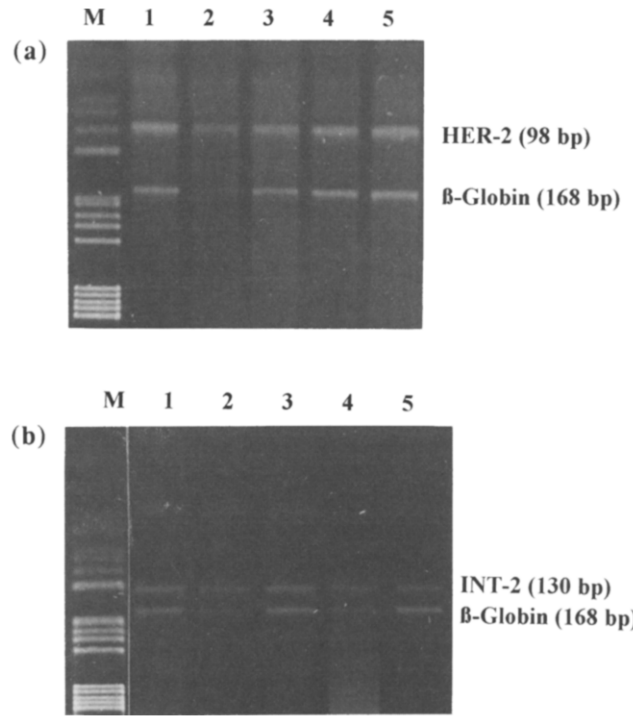

Fig. 1. Agarose gels of HER-2 and INT-2 PCR products. Lane M: 0.5 μg pBR322/HaeIII digested. (a) HER-2: lane 1: slightly amplified sample, lane 2: high copy sample, lanes 3 and 4: single copy samples, lane 5: placenta. (b) INT-2: lanes 1 and 3: single copy samples, lanes 2 and 4: low copy samples, lane 5: placenta.

Densitometry

A Hirschmann elscript 400 densitometer was used for scanning the Polaroid negatives (Type 55). The oncogene copy number was estimated from the ratio of peak areas, using the placenta ratio for normalisation. Class definition was according to Slamon *et al.* [2], i.e. single copy (amplification up to 1.50), low copy (amplification 1.51–5.00) and high copy (amplification higher than 5.00). Amplified samples could be clearly identified by densitometry up to 10-fold. Copy numbers greater than 10 could not be evaluated exactly, because the oncogene bands on the Polaroid negatives tend to be out of the linear range of the densitometer.

RESULTS

Competitive PCR systems for HER-2 and INT-2 were developed and optimised to result in equal amounts of PCR product for oncogene and reference gene for normal human placenta DNA. The validity of the quantitative PCR method was examined by comparing INT-2 amplification data obtained by dot blot and PCR systems using identical DNA samples extracted from frozen tissue. Linear regression analysis resulted in a slope of 0.98 and an intercept of 0.05 with a correlation coefficient of 0.81 (data not shown). In addition, HER-2 and INT-2 amplification analysed by dot blot in DNA samples obtained from frozen tissue was compared with quantitative PCR data analysed in DNA from paraffin-embedded tissue of the same tumour samples. Analysis of these results by linear regression is shown in Fig. 2.

PCR and dot blot data exhibit correlation coefficients of 0.74 and 0.80 for HER-2 and INT-2, respectively.

Reproducibility of the newly developed PCR method was checked by estimating single copy DNA from placental tissue and amplified DNA samples of ovarian cancer tissues using the same block on separate occasions. The PCR experiments resulted in a coefficient of variation of 10% in terms of absolute figures and the same degree of amplification was observed when 20 experiments were evaluated.

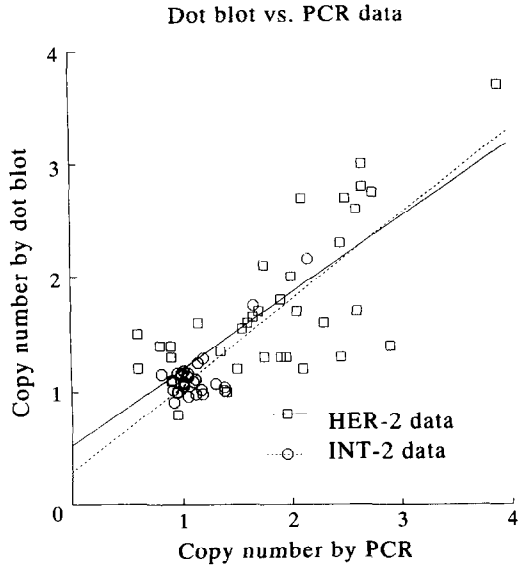

Fig. 2. Comparison of dot blot and PCR data. HER-2 and INT-2 copy numbers of 38 and 27 tumour samples, respectively, were estimated. The dot blot axis intercepts were 0.53 and 0.31, the slopes were 0.67 and 0.74 and the correlation coefficients were 0.74 and 0.80 for the HER-2 and the INT-2 systems, respectively.

Table 1. Histological types of 196 ovarian cancer samples

Histological type	*n*	%
Serous	112	57.1
Mucinous	22	11.2
Endometroid	9	4.6
Undifferentiated	25	12.8
Clearcell	10	5.1
Other	18	9.2

Table 2. Distribution of HER-2 and INT-2 copy numbers

	SC	LC	HC
HER-2	117	64	15
	59.7%	32.7%	7.6%
INT-2	158	38	0
	80.6%	19.4%	0%

SC = single copy (< 1.51 copies), LC = low copy (1.51–5.00 copies), HC = high copy (> 5.00 copies).

In a retrospective study, DNA from 196 ovarian cancer tissues that had been prepared between 1 January 1981 and 31 December 1989 was investigated for HER-2 and INT-2 copy numbers with the described PCR systems. Distribution of histological types studied in this investigation is shown in Table 1.

HER-2 and INT-2 amplification classified as single copy, low copy and high copy in 196 ovarian cancer samples is shown in Table 2. Association of HER-2 and INT-2 amplification is shown in Table 3. Of the ovarian cancer samples, 117 (59.7%) showed single copy HER-2 and 158 (80.6%) INT-2 oncogene. Amplification was noted in 79 (40.3%) and 38 (19.4%) of the cases, respectively. 64 (32.7%) ovarian cancer samples were found to have low copy HER-2 gene, 16 (7.6%) showed high amplification. No high copy INT-2 samples could be detected. Statistical analysis of data was carried out by Wilcoxon scores,

Table 3. Correlation between HER-2 and INT-2 copy numbers

		HER-2 SC	HER-2 LC	HER-2 HC
INT-2	SC	95	49	14
		48.5%	25.0%	7.1%
	LC	22	15	1
		11.2%	7.7%	0.5%

SC = single copy (< 1.51 copies), LC = low copy (1.51–5.00 copies), HC = high copy (> 5.00 copies).

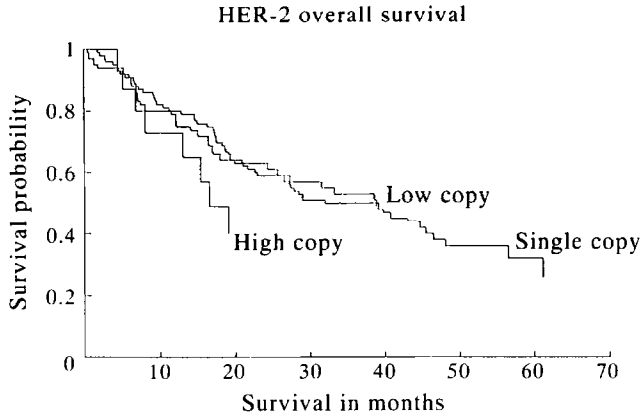

Fig. 3. Association between survival probability and HER-2 copy numbers. Single copy: < 1.51 copies; low copy: 1.51–5.00 copies; high copy: > 5.00 copies.

Kruskal–Wallis tests, χ^2 tests and Kaplan–Meier analysis (Figs 3, 4).

The mean observation period was 40 months. No significant correlation could be detected between INT-2 or HER-2 copy number and overall survival or disease-free interval. Kaplan–Meier analysis suggests that patients with higher HER-2 copy numbers have poorer prognosis. HER-2 or INT-2 amplification, did not correlate with other clinicopathological indices like grading, staging, oestrogen receptor level, progesterone receptor level and histological type. However, a statistically significant correlation of INT-2 copy numbers with preoperative CA 125 serum levels ($P = 0.03$) was found. An indirect correlation between HER-2 and INT-2 copy numbers was noted (Table 3).

DISCUSSION

In this paper a fast and non-radioactive method for the determination of oncogene amplification is described. The present technique uses much less sample material than classical methods such as Southern blot and dot blot analysis. In addition, DNA extracted for this quantitative PCR method need not be extensively purified. Data obtained by the present PCR protocol were compared with results from dot blot experiments. Dot blot was chosen as the reference method since it is faster, needs less experimental steps and is less prone to error than Southern blot.

Previous protocols of quantitative PCR describe methods using internal standards [19]. The use of a reference gene in differential PCR has already been reported [20]. In the present experiments, the β-globin gene served as a reference for PCR runs and the β-actin gene was used as a reference for dot blotting. These reference genes were used to account for partial ploidy or amplification of the selected reference gene in the tumour cells. Linear regression analysis of PCR and dot blot data showed good correlation when identical DNA samples were used. Comparison between results of dot blot experiments run on DNA extracted from frozen tissue samples and data of PCR investigations carried out in DNA extracted from paraffin-embedded tissue sections corroborates results obtained in identical DNA samples. To minimise PCR artefacts, single copy, high copy and negative controls were included in each PCR run. The PCR method used in the present experiments showed good reproducibility when the same block of tissue was investigated on separate occasions and the same degree of amplification was observed by these controls. These experiments showed that paraffin-embedded tissue samples can be reliably analysed by quantitative PCR for oncogene amplification.

In 95/196 (48.5%) cases no HER-2 and INT-2 oncogene amplification could be detected. One or both oncogenes were amplified in 101/196 (51.5%) of cases. Among these only 16 (8.2%) showed amplification of both oncogenes having low copy INT-2 and low or high copy HER-2 oncogene. In the majority (85/101) of this group only one of either oncogene was found to be amplified, indicating a negative correlation between HER-2 and INT-2 copy numbers. This observation was described for mammary carcinomas previously [12, 21, 22]. Present results corroborate previous studies on HER-2 amplification [4, 23, 24]. However, in contrast to another report [4], no association between HER-2 amplification and survival was noted in the present investigation. Further studies are necessary to investigate this observation in more detail.

It was interesting to note that no high INT-2 copy numbers were found. This is different from reports on mammary carcinoma where 3 to 10% INT-2 high copy samples were noted [12, 25, 26]. Various observations in mammary tumours indicate that INT-2 is a proliferation marker since amplification of this oncogene correlates with steroid receptor levels, recurrence and lymph node status [21, 26]. In addition, a significant correlation between INT-2 copy numbers and preoperative CA125 serum levels was found (data not shown).

Present data combine to suggest that this quantitative PCR method is a valuable technique for the evaluation of HER-2 and INT-2 amplification in paraffin-embedded tissue samples for retrospective and prospective studies.

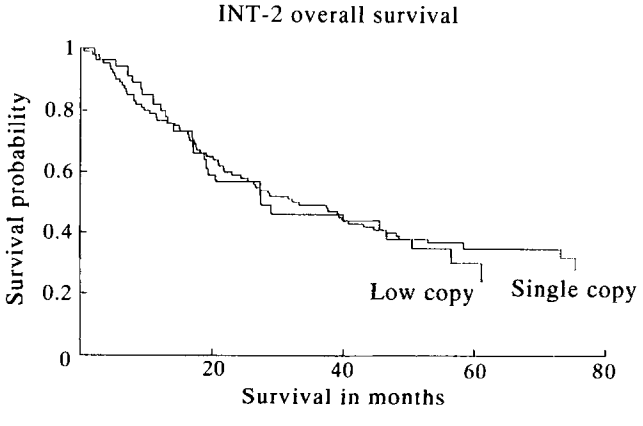

Fig. 4. Association between survival probability and INT-2 copy numbers. Single copy: < 1.51 copies; low copy: 1.51–5.00 copies.

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Phase II Study of Cystemustine in Metastatic Colorectal Carcinoma

A Trial of the EORTC Clinical Screening Group

P. Kerbrat, A. Adenis, P. Rebattu, H. Roche, B. Chevallier, Ph. Chollet, I. Krakowski, M.A. Lentz and P. Fumoleau for the EORTC Clinical Screening Group

27 patients with metastatic colorectal carcinoma were treated, every 2 weeks, with 60 mg/m² cystemustine, a new chloro-2-ethyl nitrosourea derivative. Haematological toxicity was the major side-effect including neutropenia and thrombocytopenia. We did not observe any complete or partial response. Cystemustine, with this dose and this schedule, has no activity in colorectal cancer.
Eur J Cancer, Vol. 29A, No. 11, pp. 1597–1599, 1993.

INTRODUCTION

COLORECTAL CARCINOMA causes 10–15% of morbidity encountered in western countries. This particular type of tumour is resistant to most of the chemotherapeutic drugs, except for fluoro-pyrimidine derivatives, especially 5-fluorouracil [1]. These drugs have a consistent antitumour activity in colorectal tumours. Interestingly, some studies report activity of another class of drugs, the nitrosoureas, CCNU, BCNU and methyl CCNU [2].

In order to investigate the therapeutic value of these results, the EORTC Clinical Screening Group performed a phase II trial using a new nitrosourea compound cystemustine.

PATIENTS AND METHODS

Patient eligibility was defined by a measurable metastatic colorectal tumour within a 3-months period prior to the beginning of treatment, a WHO performance status of 0–1, a normal white blood cell and platelet count and normal renal and