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HER2 gene amplification in patients with prostate cancer: Evaluating a CISH-based method

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Abstract. Prostate cancer (PCa) is one of the most widespread malignancies in the world. The role of the human epidermal growth factor receptor 2 (HER2) in the pathogenesis and progression of human PCa remains poorly understood. In contradiction with breast cancer, studies on HER2 overexpression and gene amplification in PCa have produced varying results, although the HER2 oncogene has been implicated in the biology of numerous tumor types, and serves as a prognostic marker and therapeutic target in breast cancer. Technical challenges are considered the main reasons for data discrepancies. Amplification of the HER2 gene has previously been reported in PCa, in which it was associated with tumor progression. The present study aimed to evaluate the prevalence and clinical significance of HER2 amplification in PCa. A total of 32 biopsy samples obtained from human prostate adenocarcinomas were evaluated by chromogenic *in situ* hybridization (CISH) to determine the frequency of patients with HER2 gene amplifications. High copy numbers of HER2 were detected in 19 of the prostate tumors analyzed. The results of the present study suggested that, in patients without amplification of HER2, high levels of prostate-specific antigen or a high Gleason score were not significantly correlated with a high pathologic stage. Furthermore, amplification levels of the HER2 gene were directly associated with pathologic stage in patients with PCa. Therefore, the potential use of HER2 as a prognostic factor or therapeutic target for PCa warrants further study.

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Abbreviations: PCa, prostate cancer; HER2, human epidermal growth factor receptor 2; CISH, chromogenic *in situ* hybridization; PSA, prostate specific antigen; FISH, florescence *in-situ* hybridization

Key words: prostate cancer, human epidermal growth factor receptor 2, amplification, gene, chromogenic *in situ* hybridization

Introduction

Prostate cancer (PCa), a common non-skin, sex-limited cancer, is the second cause of cancer-associated mortality (after lung cancer) in the USA (1,2) and the second most prevalent cancer among Iranian men (3). Worldwide, PCa is the second most commonly diagnosed cancer and, according to the International Agency for Research on Cancer's GLOBOCAN 2012 (4) database, it is the fifth leading cause of cancer-associated mortality in men. The incidence of PCa is increasing worldwide, although there is a marked variation in its incidence among different regions (5). The clinical configuration of PCa has noticeably altered over the past few years. As a localized disease, it is easily treated by radical radiation therapy or a prostatectomy; however, if the tumor becomes malignant, it transforms into a life-threatening disease (6).

The progression and application of novel high-resolution technologies has enhanced the detection of genomic alterations, enabling elucidation of the complex nature and heterogeneity of PCa (7). The differentiation of PCa tumors is typically based on the serum expression levels of prostate-specific antigen (PSA), although, in certain cases, PSA levels do not accurately reflect tumor burden (8). Previous studies have identified a number of genetic, epigenetic and environmental risk factors for PCa (9-11). Among them, genetic aberrations and chromosomal changes have been suggested to serve a significant role in the development and progression of PCa (12). At present, >50 PCa susceptibility loci have been identified

using genome-wide association studies (13,14). The emerging picture of the genomic complexity of PCa includes frequent large-scale genomic rearrangements (15), gene fusions (16,17), genetic deletions (15) and gene amplifications (18).

Gene amplification, which may occur due to an increase in copy number of certain regions of chromosomes, has been identified in several malignancies, including PCa (18,19). Previous studies have reported that the genetic duplication of various genes was associated with PCa malignancy, including androgen receptor (20), enhancer of zeste homolog 2 (21), eukaryotic translation initiation factor 3 (22), calcium-activated potassium channel subunit α-1 (23), minichromosome maintenance complex component 7 (24), prostate leucine zipper (25) and hypoxia-inducible factor 1 (26).

Human epidermal growth factor receptor 2 (HER2) is a member of the class I receptor tyrosine kinase family and has substantial homology to epidermal growth factor receptor, HER3 and HER4 (27). HER2 overexpression and/or gene amplification occur in a variety of human epithelial tumors, particularly in breast cancer, in which the receptor and its gene have been investigated extensively (28). Conversely, the significance of HER2 overexpression and gene amplification in PCa remains controversial. Previous studies have used immunohistochemical analysis to evaluate HER2 protein expression in primary prostate specimens, demonstrating expression rates ranging from 0-100% (29-31). Therefore, the exact prevalence of HER2 gene amplifications in primary PCa remains unknown, likely owing to the wide range of antibodies and methods used in these studies (32,33).

The HER2/neu proto-oncogene, which is located on chromosome 17 (OMIM: 164870), encodes a transmembrane tyrosine kinase growth factor receptor (34), whose overexpression was shown to be involved in the development of various types of human cancer, including non-small-cell lung cancer, colon cancer and breast cancer, and may have prognostic value (35,36). Apparent chromosome 17 polysomy, defined by increased chromosome enumeration probe 17 (CEP17) signal number, is a common genetic aberration in breast cancer and represents an alternative mechanism for increasing HER2 copy number (37). However, the prognostic value of HER2/neu amplification in PCa remains controversial (38).

Chromosomal aberrations associated with PCa have been evaluated using various techniques, including classical cytogenetics (39), loss of heterozygosity analysis (40), fluorescence *in situ* hybridization (FISH) (41) and, most commonly, comparative genomic hybridization (CGH) (42). Although the criteria for amplification have varied between studies, they have implicated several chromosomal regions, such as 6q, 8p, 10q, 13q, 16q and Xq, that may harbor genes involved in the tumorigenesis of PCa (24,43).

The present study aimed to investigate the frequency of HER2 amplification in prostate biopsies from Iranian (Tehran province) patients using chromogenic *in situ* hybridization (CISH), which permits the rapid analysis of a large number of tumors (44). Although the FISH method has been verified for the histological analysis of tissues, the evaluation of tumor morphology using FISH is challenging and the fluorescence fades quickly (45). These limitations may be overcome by CISH, which enables visualization of the amplification product along with morphological features (46). Furthermore, CISH technology is superior to high-throughput HER2 genetic testing due its speed, although FISH remains the method of choice for rapid low-throughput HER2 genetic testing.

Materials and methods

Clinical specimens. The present study was approved by the Ethics Committee of Tehran University of Medical Sciences (Tehran, Iran). Suitable patients from the oncology wards or outpatient clinics of Imam Khomeini Hospital (Tehran, Iran) were approached for participation in the study, and informed consent was obtained. Inclusion criteria for the study were a PSA level of >4, a diagnosis of progressive prostate cancer, an age of >54 years, a Gleason score of >2 (47) and the male gender. Formalin-fixed, paraffin-embedded (FFPE) specimens were obtained from 32 consecutive PCa patients who underwent surgery between May 2013 and February 2015. Adjacent normal tissue was used as a control. To account for tumor heterogeneity, a minimum of 3 cylindrical core biopsies, 0.6 mm in diameter, were harvested from different regions of each tumor. A total of 15 tissue sections (2-mm thick) were sliced from each paraffin-embedded tumor block and mounted onto glass slides. The first tissue section was stained with hematoxylin and eosin and visualized under a light microscope to ascertain the region of interest. In all cases, a serum sample was measured by Elecsys® total PSA and free PSA kits (Roche Diagnostics, Basel, Switzerland).

Pathological stage. The stage (extent) of prostate cancer is one of the most important factors in choosing treatment options and predicting prognosis. The stage is based on the prostate biopsy results (including the Gleason score), the blood PSA level at the time of diagnosis, the results of any other exams or tests that were performed to determine metastasis and the pathological stage post-surgery. There are 4 categories for describing the local extent of a prostate tumor, ranging from T1 to T4 (48).

CISH. CISH was performed according to a previously described protocol (49) with minor modifications. Briefly, 2-mm thick archival FFPE tissue sections were deparaffinized and dehydrated in 0.1 mol/l Tris-HCl (pH 7.3) in a temperature-controlled microwave oven at 92˚C for 10 min. Subsequently, the sections were allowed to cool for 20 min, followed by washing with phosphate-buffered saline (PBS) for 3-4 min at 37˚C. The slides were then dehydrated with graded ethanol, rinsed in saline sodium citrate (pH 7.2) for 5 min at room temperature and air-dried. Enzymatic digestion was performed by incubating the sections with pepsin solution (dilution, 100:l) for 10 to 15 min at room temperature. The slides were then washed with PBS and dehydrated with graded ethanol. The ready-to-use digoxigenin-labeled HER2/neu probe (Zyto Dot 2C SPEC HER2/CEN 17 Probe kit; Zyto Vision GmbH, Bremerhaven, Germany) was applied onto the slides. The presence of certain nucleic acid sequences in cells or tissue can be detected with *in situ* hybridization using labeled DNA probes. The hybridization results in duplex formation of sequences present in the test object and the specific gene probe. The ZytoDot 2C SPEC HER2/CEN 17

Table I. Prostate cancer patients with or without HER2 amplification.

Patient ID	Age	Situation	PSA	Pathologic grade	Gleason score	Green signal	Red signal	Green/red
P-709	55	L	5.1	2	4	271	116	2.31
P-436	82	R	8.1	3b	5	410	134	3.05
P-1139	56	L	8.2	2	4	201	83	2.42
P-100	65	L	9.2	3a		355	130	2.73
P-326	61	L	9.3	4b	6	296	90	3.28
P-435	82	R	6.1	3a	5	168	75	2.24
P-1101	67	R	195	4b	7	401	134	2.99
P-564	66	L	10.9	3a	6	201	68	2.95
P-62	69	L	32.8	3a	5	278	116	2.39
P-27	83	L	9.3	3a	5	180	71	2.53
P-63	61	R	14.8	3a	5	214	95	2.25
P-425	82	L	16.2	3a	5	220	75	2.93
P-599	75	R	74.3	4b	7	224	88	2.54
P-13	83	R	16.4	4b	6	485	181	2.67
P-68	70	L	156.2	4b	7	182	73	2.49
P-80	70	R	9.2	4	6	465	193	2.41
P-69	66	L	19.2	4	7	459	186	2.46
P-137	78	R	20.8	4	6	150	68	2.22
P-162	75	L	33.1	4	6	421	166	2.53
P-691	65	L	4.95	2	4	444	256	1.73
P-993	61	L	69.8	4b	8	212	165	1.28
P-1084	82	L	45.7	4a	7	-	-	-
P-806	78	L	6.1	4b	7	229	112	2.04
P-856	80	L	78.3	4a	6	240	123	1.95
P-27	83	L	8.1	3a	5	221	110	2.00
P-150	68	R	8.9	4b	6	-	120	-
P-439	82	L	6.1	3a	5	160	79	2.02
P-174	57	R	5.5	2	4	112	60	1.80
P-12	71	L	18.6	3c	5	478	233	2.05
P-37	77	L	140.1	4	6	110	59	1.86
P-83	58	L	7.3	4	5	165	86	1.91
P-164	74	R	4.4	4	5	596	281	2.12

HER2 amplification was considered when the green/red ratio was >2.2. HER2, human epidermal growth factor receptor 2; PSA, prostate-specific antigen; L, left; R, right.

Table II. P-values for the associations between the mean age, serum levels of PSA, green/red ratio and Gleason score of patients without human epidermal growth factor receptor 2 amplification.

Parameter	Age	PSA	Green/red	Gleason
Age	-	0.23	0.046	0.253
PSA	-	-	0.228	0.004
Green/red	-	-	-	0.941

PSA, prostate-specific antigen.

Probe kit uses the ZytoDot 2C SPEC HER2/CEN 17 Probe EmaNOF. The probe contains digoxigenin-labeled polynucleotides, which target sequences of the HER2 gene and DNP-labeled polynucleotides, which target alpha-satellites of the centromere of chromosome 17. Duplex formation of the labeled probe can be visualized using primary (un-marked) antibodies, which are detected by secondary polymerized enzyme-conjugated antibodies. The enzymatic reactions of the substrates leads to the formation of strong permanent red and green signals that can be visualized by light microscopy using a 40X dry lens. The slides were denatured on a hot plate for 3 min and hybridization was performed overnight at 37˚C. Following hybridization, the slides were washed with 0.5 ml standard saline citrate for 5 min at 75˚C, followed by three washes with PBS containing 0.2% Tween-20 at room temperature. Prostate tissue sections were lightly counterstained with hematoxylin then embedded, and the tissues were analyzed under a light microscope. Amplification was defined when the HER2/chromosome 17 centromere (CEP17) ratio was >2.2.

Table III. Associations among tumor position, pathological stage, age, serum levels of PSA, green/red ratio and Gleason score in patients without human epidermal growth factor receptor 2 amplification.

Parameter	Age	PSA	Green/red	Gleason score
Tumor position				
Left	73.7±9.3	38.4±45.2	1.88±0.23	5.8±1.2
Right	66.33±8.61	6.1±2.5	1.97±0.16	5±1
P-value	0.15	0.009	0.453	0.240
Pathologic stage				
2	61±5.6	5.22±0.38	4	1.76
3a	82±0.70	7±1.41	2	5
3c	71	18.6	2.05	5
4	69.66±10.21	50.43±77.58	1.96±0.14	5.33±0.5
4a	81±0.71	62±23.05	1.95	6±0.71
4b	69±8.54	28.27±36.00	1.66±0.54	7±1
P-value	0.142	0.749	0.873	0.057

Data are presented as the mean ± standard deviation. PSA, prostate-specific antigen.

Table IV. P-values for the associations between the age, serum levels of PSA, green/red ratio and Gleason score of patients with human epidermal growth factor receptor 2 amplification.

Parameter	Age	PSA	Green/red	Gleason
Age	-	0.248	0.571	0.522
PSA	-	-	0.404	<0.001[a]
Green/red	-	-	-	0.62

[a]Correlation is significant at the 0.01 level (two-tailed). PSA, prostate-specific antigen.

Statistical analysis. Red/green signals were counted manually. Data analysis was performed using SPSS software version 18 (SPSS, Inc., Chicago, IL, USA). Scale variables were analyzed for normality using the Kolmogorov-Smirnov test. Group comparisons of continuous variables were conducted using the independent-samples *t*-test. When a variable was non-normally distributed, Mann-Whitney or Kruskal-Wallis non-parametric tests were performed. GraphPad Prism version 5.0 software for Windows (GraphPad Software, La Jolla, CA, USA) was used to illustrate the data through graphs. Data are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Associations among age, PSA levels, green/red ratios and Gleason scores in patients with or without HER2 amplification. For statistical analysis, patients were divided into two groups consisting of patients with or without HER2 amplification. Variables were assessed within each group and the results are presented in Tables I-III. The demographic data of patients with PCa with and without HER2 amplification are shown in Table I. There were no associations among the serum levels of PSA, green/red ratios (Fig. 1) or Gleason scores in patients without HER2 amplification.

Amplification levels of the HER2 gene are directly associated with pathological stage in patients with PCa. There was a weak association between the green/red ratio and age in patients without HER2 amplification (P=0.046; Table II), thus suggesting that younger patients exhibited a lower tendency for HER2 amplification. In addition, a positive correlation (P=0.004) was observed between the serum levels of PSA and Gleason score (Table II). The associations between the green/red ratio and PSA levels or Gleason score were not significant (P=0.228 and 0.941, respectively; Table II).

For the analysis, patients were divided into two separate groups based on the level of HER2 gene amplification (i.e., with and without amplification). The first group consisted of 19 patients and the second group consisted of 13 patients. A high level of HER2 gene amplification was considered when the HER2/CEP17 ratio was >2.2 (Fig. 1).

The mean ages were 73.7 and 63.3 years for patients with left- and right-side tumors, respectively, which were not significantly different (P=0.15; Table III). With the exception of the serum levels of PSA (P=0.009), there were no significant differences in any of the parameters (green/red ratio and Gleason score) between patients with left- and right-sided tumors. The mean Gleason score among patients was 5, and the Gleason score showed no association with the pathologic stage (P=0.303; Table III). The tumors were composed of different Gleason scores, but were between stages T2 and T5 (Table III). Similar to the patients without HER2 amplification, there was a significant association between the Gleason score and serum levels of PSA in patients with HER2 amplification (P<0.001; Table IV). However, there was no significant association between the other parameters in patients with HER2 amplification (P>0.05; Table IV). The tumor position was not significantly associated with the mean age, PSA levels, green/red ratio or Gleason score of patients with HER2

Table V. Associations among tumor position, pathologic stage, age, serum levels of PSA, green/red ratio and Gleason score in patients with human epidermal growth factor receptor 2 amplification.

Parameter	Age	PSA	Green/red	Gleason score
Tumor position				
Left	68.0±9.24	28.4±43.5	2.64±0.3	5.5±1.0
Right	74.75±8.0	43±65.25	2.55±0.33	5.89±0.83
P-value	0.75	0.643	0.382	0.290
Pathologic stage				
2	55.5±0.71	6.55±2	2.36±0.08	4
3a	73.83±9.67	15±9.45	2.58±0.3	5.17±0.4
3b	82	8	3.05	5
4a	72.25±5.32	20.55±9.7	2.4±0.15	6.25±0.5
4b	71.2±8.32	90.24±83.04	2.79±0.33	6.6±0.55
P-value	0.095	0.078	0.123	0.002

Data are presented as the mean ± standard deviation. PSA, prostate-specific antigen.

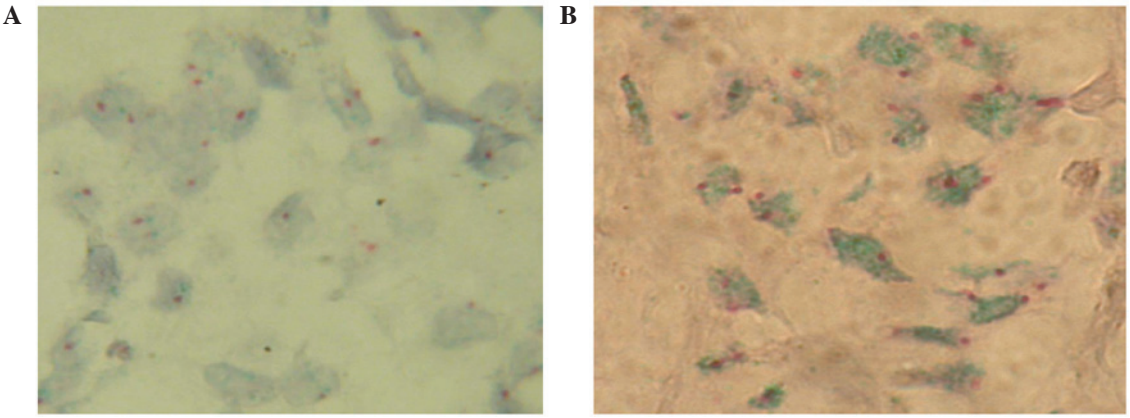

Figure 1. Human epidermal growth factor receptor 2/neu oncogene gene status in prostate carcinomas, as detected by chromogenic *in situ* hybridization. Green signals correspond to HER2, and red signals correspond to (magnification, x100). (A) HER2 gene non-amplified. (B) HER2 gene amplified. A high level of HER2 gene amplification was detected when the HER2/CEP17 ratio was >2.2. HER2, human epidermal growth factor receptor 2; CEP17, chromosome 17 centromere.

amplification, although a significant correlation was observed between the Gleason score and pathologic stage (P=0.002; Table V).

Discussion

DNA ploidy has been accepted as a significant predictor of prognosis in patients with PCa (50). In the present study, amplification and overexpression of HER2 was demonstrated in patients with PCa, which has previously been associated with cancer progression, a poor prognosis and development of androgen independency (51). HER2 status is routinely assigned using *in situ* hybridization to assess HER2 gene amplification, but interpretation of *in situ* hybridization results may be challenging in tumors with chromosome 17 polysomy or intratumoral genetic heterogeneity. Apparent chromosome 17 polysomy, defined by increased CEP17 signal number, is a common genetic aberration in breast cancer and represents an alternative mechanism for increasing HER2 copy number. Elevated CEP17 count (polysomy) has been linked with adverse clinicopathologic features and HER2 overexpression, although there are numerous discrepancies in the literature (37). HER2 overexpression and/or amplification are recurrently reported in numerous tumor types, and have been shown to have significant therapeutic implications in patients with cancer (33). A meta-analysis of 5,976 patients demonstrated that HER2/neu overexpression was associated with mortality and recurrence in patients with PCa (52). Furthermore, it has been suggested that HER2 overexpression at the protein level is significantly associated with the amplification of HER2 (53). FISH is considered the gold standard method for detecting gene amplification, and has been reported to be more accurate than flow cytometry and immunohistochemistry (54). An increasing number of authors have employed the CISH method for determining gene amplification in various types of cancer (37,55,56). CISH is a recently developed technique in which the DNA probe is located based on an immunoperoxidase reaction. This method is similar to FISH, although it does not involve the use of fluorescence microscopy. In addition, FISH signals fade within a few weeks and the FISH results must be recorded using

expensive digital systems, which is not required for CISH staining. Owing to its resemblance to immunohistochemistry staining (57), CISH is also easier to interpret by pathologists who are not trained in fluorescence microscopy. Furthermore, in previous studies, CISH was observed to be well-correlated with FISH (46,58-60).

The present study used standard CISH to demonstrate that HER2 was amplified in Iranian (Tehran province) patients with PCa. Notably, HER2 amplification was observed in >50% of patients. Similarly, using a FISH technique, a study on 44 patients with PCa demonstrated 53 and 80% low copy amplification in non-metastatic and metastatic samples, respectively (61). The results of the present study were consistent with previous studies, in which HER2 amplification was reported in 44 and 41% of 62 and 113 Americans, respectively, using the FISH method (54,62), whereas another analysis reported no HER2 amplification (63). Furthermore, in a previous study, FFPE tissue blocks from 88 patients demonstrated a minor amplification rate of 9.3% (8/88 cases) (64). Similarly, Qi *et al* (53) used a FISH method and demonstrated that only 5.8% of Chinese patients with PCa had such a genetic alteration, and an investigation of 93 cancer samples showed that 6.5% had low levels of HER2 amplification, which was co-amplified with the topoisomerase (DNA) II α gene (65). These conflicting results may exist due to variation in the sample size and method used, or as a result of genetic heterogeneity. Furthermore, the findings may suggest that the CISH method is superior to FISH for HER2 detection in PCa samples. CISH has also been utilized for detection of copy number variation in the HER2/neu gene (66). In addition, the accuracy and reproducibility of CISH has been demonstrated in a previous study of breast carcinoma, in which the authors suggested that CISH may be regarded as a practical alternative for FISH (67). Other studies have considered this matter and proposed that CISH is a viable alternative to FISH and had similar properties; for example, both are *in situ* hybridization techniques and directly visualise the number of gene copies present in the nucleus, but CISH is cheaper and it produces a stable record of the slide that can be interpreted with a light microscope in the background of the tumour histopathology (68). Permanent staining and the absence of a fluorescent dye make CISH a suitable replacement for FISH (69). In addition, its usability, relative inexpensiveness and speed make CISH more attractive than FISH for assessing HER2 amplification/overexpression (59,70).

In conclusion, to the best of our knowledge, the present study is the first to report the amplification of HER2 in Iranian patients (Tehran province) with PCa. Furthermore, it was demonstrated that there were no associations among the serum levels of PSA, green/red ratios or Gleason scores in patients without HER2 amplification. Conversely, there was a weak correlation between the green/red ratio and age in these patients (P=0.046), which suggested the tendency for younger patients to exhibit lower levels of HER2 amplification. Notably, there was no association between the green/red ratio and pathologic stage of patients without HER2 amplification (P=0.873), although the increasing trend suggested that clinicians may consider Herceptin as a drug of choice for patients with PCa. In addition, there was no association between PSA levels (P=0.749) or Gleason score (P=0.057) and pathologic stage in patients without HER2 amplification. In patients with HER2 amplification, there was a significant association between the Gleason score and the serum level of PSA (P<0.001). However, there was no significant association between the other parameters in patients with HER2 amplification (P>0.05). The tumor position was not significantly associated with the mean age, PSA level, green/red ratio or Gleason score of the patients with HER2 amplification, although a significant correlation was observed between the Gleason score and pathological stage (P=0.002; Table V). Finally, the present study confirmed the results of previous studies, which suggested that the CISH method may be considered a valuable replacement for FISH. Further studies involving PCa samples are required in order to validate the results of the present study.

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