Role of low hemoglobin level in predicting response to neoadjuvant chemotherapy in breast cancer and its correlation with p53, bcl-2 and VEGF – a prospective clinical study

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IN BRIEF

Neo-adjuvant chemotherapy (NACT) is an integral part of multi-modality approach in the management of locally advanced breast cancer (LABC). It is required both for the local control (to ensure microscopically free margins during surgery) and distant or systemic control [1-6]. Development of resistance to chemotherapeutic agents is a major and evolving problem. Various markers like P-Glycoprotein, tumor suppressor gene p53, apoptotic markers(Bcl-2, Bax) have been studied to predict the response to NACT[1].

Key words: Hemoglobin, breast cancer, neoadjuvant, chemotherapy

Introduction

Few studies suggest that the response to cyclophsphamide which is a highly oxygen dependent cytotoxic drug is dependent on Hemoglobin level[7-9]. The prognostic impact of anemia in cervical cancers is well established. In a recent meta-analysis, anemia was found to be an independent prognostic factor for increased death rates in various solid tumors (head and neck, lung, prostate) and lymphoma[7].The relation between low Hb levels and a low oxygenation status of malignant tissues could be demonstrated in several studies[10,11,12,13,14]. Tumor hypoxia is a direct consequence of structural abnormalities of the microvasculature and functional impairments of the microcirculation and results from either limited pO2 diffusion (chronic hypoxia) or limited perfusion (acute hypoxia)[11]. Singer et al demonstrated with breast cancer cell lines that diminished mitochondrial energy generation (due to tumor hypoxia) was related to malignant progression[15,16].

Poor oxygenation of residual and accelerated repopulating tumor cells may have severe effects on tumor cell biology. Hypoxia is one of the reason for genetic instability and the development of mutations in malignant tissues[10,17,18]. Such mutations could affect genes encoding for apoptotic cell death. An example of such a gene is p53 tumor suppressor gene, which induces apoptosis in hypoxic tumor cells. Genetic alterations promoted by hypoxia could result in mutant p53 i.e a loss of functional p53 tumor suppressor gene and therefore in a loss of apoptotic potential in hypoxic tumor cells[10,19]. Another form of genetic alteration in hypoxic tumor cells is overexpression of the apoptosis inhibitor protein Bcl-2. According to Graeber et al. an overexpression of this protein could be demonstrated especially in hypoxic tumor cells[10,19].

Normal breast development is controlled by a balance between cell proliferation and apoptosis, and there is strong evidence that

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tumor growth is not just a result of uncontrolled proliferation but also of reduced apoptosis[20]. A large number of anti-cancer agents with widely differing modes of action have been demonstrated to induce apoptosis in vitro, suggesting this as a significant final common pathway for exerting their clinical effects. Mechanisms that suppress apoptosis may be important in the development of intrinsic and acquired resistance to cytotoxic drugs[1,4].

Hypoxia of tumor cells is the most stimulating factor for release of vascular endothelial growth factor[10,17]. VEGF is the most important molecule for angiogenesis[10,17]. In consequence, hypoxic malignant tissue could more rapidly develop a sufficient vascularisation than normoxic tumors. Angiogenesis is necessary for the growth and invasiveness of primary tumors and is integral part of cascade of biologic events involved in tumor metastasis[21].

One of the major problem with NACT is chemoresistance. It is therefore vital to assess the response to NACT in order to tailor regime for a particular patient to predict the chemoresistance[1]. The clinical response along with complete pathological response is still considered a surrogate marker of response against which all other predictive markers are compared. Various markers like P-Glycoprotein, tumor suppressor gene p53, apoptotic markers(Bcl-2, Bax) have been studied to predict the response to NACT. The need to have a reliable and inexpensive predictor of response in third world scenario can not be overemphasized since majority of patients present late and the resources are limited and scarce.

Low hemoglobin level causes hypoxia in tumor cells, which in turn reduces apoptosis by causing genomic instability i.e overexpression of anti apoptotic gene Bcl-2 and decreased expression of functional tumor suppressor gene p53. Tumor hypoxia also causes increased angiogenesis and thus increased expression of VEGF. Low hemoglobin level, therefore may be utilized as a cost effective and reliable predictor of response to NACT. Against this background a prospective study was contemplated with following aims and objectives;

1. To correlate hemoglobin level with tumor suppressor gene p53, apoptotic inhibitor Bcl-2 and vascular endothelial growth factor VEGF.
2. To correlate expression of tumor suppressor gene p53, apoptotic inhibitor Bcl-2 and vascular endothelial growth factor with response to neoadjuvant chemotherapy.
3. To ascertain whether the low hemoglobin level could be utilized as a reliable predictor of response to neoadjuvant chemotherapy.

Methods

152 FNAC proven cases of locally advanced breast carcinoma according to AJCC (American Joint Committee On Cancer) classification were included in the study.

A thorough clinical and ultrasonographic examination (USG) of all the patients including the opposite breast was performed to stage the disease accurately. A core biopsy using a tru-cut needle was performed for immuno-histochemical estimation of the apoptotic inhibitor i.e. Bcl-2, tumor suppressor gene p53 and vascular endothelial growth factor (VEGF) before initiating the chemotherapy. Routine and metastatic work up was done including complete blood examination (total blood count including hemoglobin level, platelet count), chest radiograph, ECG (Echocardiography when ECG had a positive finding), liver function tests, Bone Scan, USG abdomen, KFT (Kidney function tests).

Patients were subjected to three cycles of FAC regime containing cyclophosphamide , adriamycin , 5-fluorouracil at an interval of three weeks. Before each cycle the patients were clinically and sonologically examined for the breast tumor size, axillary lymph node status & appearance of systemic metastasis. Patey's modified radical mastectomy was performed three weeks after the last cycle and the mastectomy specimen was examined for pathological response, resected margins,

axillary lymph nodes, Bcl-2, p53 and VEGF expression(post NACT).

On the basis of Hb level patients were divided into two groups. Those with Hb level less than or equal to 11 g/dl were included in low Hb group (anemic), while those with more than 11 g/dl were included in normal Hb group(non anemic).

The pathological tumor response was evaluated by size measurement at the time of tumor resection macroscopically and by detecting tumor cell existence (or not) microscopically.

Clinical responders

Were defined as patients with a complete (CR) or partial response (PR) [CR: complete resolution of tumor, PR>50% regression in maximum diameter of initial tumor] after 3 cycles of NACT. Non-responders were patients with a minimal response (MR<50% regression in maximum diameter of initial tumor), no change (NC) or local progression. Pathological complete response (pCR) was defined as absence of any gross or microscopic evidence of residual tumor in the mastectomy specimen i.e. absence of residual invasive or in situ disease following NACT. Its assessment was done irrespective of the clinical response status. Clinical response was taken into consideration for statistical analysis as the pCR was observed in only 20(19%) patients(n=105).

Immunohistochemical methods

Methods for antigen retrieval

Biopsy specimen was preserved in buffered formalin solution. Five-micron sections were prepared from paraffin embedded blocks on poly-l-lysine coated glass slides. Sections were deparaffinized in xylene for 15 minutes and hydrated in alcohol for 15 minutes. Further, incubation was done in 0.3% hydrogen peroxide in methanol solution for 45 min. The slides were washed with citrate buffer and kept in a water bath at 90–95°C for 45 minutes for antigen retrieval.

Methods FOR BCL-2 expression

Sections were washed with Tris buffer saline (TBS) solution and incubated with blocking antibodies (DAKO monoclonal mouse antihuman Bcl-2 oncoprotein for Bcl-2 expression) at 37°C. Sections were washed with TBS solution. Incubation with avidin-biotin complex (ABC) was done at 37°C for one hour and washed with TBS solution. 3,3 Diaminobenzidine tetra hydrochloride solution applied for 3–5 min. Counter-staining with haemotoxylin solution done for 3–5 min. Sections were washed with distilled water, air dried and mounted using DPX mountant.

Methods for P53 and VEGF expression

Sections were washed with Tris buffer saline (TBS) solution and incubated with blocking antibodies (Santa cruz monoclonal mouse antihuman p53 antibody for p53 expression and monoclonal VEGF antibody for VEGF expression) at 4°C overnight. Incubation with biotinylated secondary antibody was done at 37°C for 45 minutes. Incubation with streptavidin-HRP conjugate was done for another 45 minutes[3,3]. Diaminobenzidine tetra hydrochloride solution applied for 3–5 min. Counter-staining with haemotoxylin solution done for 3–5 min. Sections were washed with distilled water, air dried and mounted using DPX mountant.

For Bcl-2, positive controls were the mantle zone of lymphoid follicles, for p53 the positive controls were normal breast cancer tissue and for VEGF the positive controls were normal placental tissue . The negative controls for Bcl-2, p53 and VEGF were the test slides without primary antibody.

The pattern of positive staining for bcl-2 and VEGF was cytoplasmic.

The pattern of positive staining for p53 was nuclear.

The primary antibody for bcl-2 was procured from DAKO.

The primary antibody for p53 and VEGF were procured from Santa cruz.

Bcl-2 Monoclonal Mouse Anti-Human code no. M 0887.

Code no. for p53 was (DO-1)sc126.

Code no. for VEGF was A 20 sc152.

Dilution for Bcl-2 was 1:40, for p53 1:50 while for VEGF dilution was 1:100.

The results were interpreted on the basis of following criteria:

Percentage of cells showing immune bodies; <10%: score = 0, 10–25%: score = 1, 25–50%: score = 2, >50%: score = 3

Score 0 and 1 were taken as negative results while score 2 and 3 were taken as positive.

The intensity of staining was also assessed and it was found that staining intensity correlated closely with percentage of positive cells showing immune bodies and a single index i.e. percentage of positive cells was used for analysis.

Statistics

Descriptive studies were performed with SPSS version 10. The significance of response assessed using paired t-test. Significance of correlation between various variables assessed using chi-square test and coefficient of correlation was calculated by Pearson correlation coefficient.

Results

152 patients of LABC were included in the study with mean age being 45.36 years (range 26-65 years) and 80 (52.6%) patients were premenopausal. The mean tumor size before NACT was 7.24 cm. 61 patients had N1 disease, 81 patients presented with N2 disease while 10 patients had N3 disease in the axilla.

The clinical response was assessed using stringent World Health Organization (WHO) criteria and reduction in mean tumor size after three cycles of NACT was found to be statistically significant ($p < 0.05$). 105 patients (69%) patients (n = 152) were responders [*complete response in 20 and partial response in 85 patients (n =105)*] while the rest 47(30.9%) were non-responders.

Significant clinical response was observed in the axillary lymph node status after NACT. Of 61 N1 patients 38(62.2%) patients showed complete response i.e. they were down staged to N0, 14(22.9) patients remained N1, while 9(14.7%) patients progressed to N2 disease. Amongst the patients with N2 disease (n = 81), 20(24.6%) were downstaged to N0, 32(39.5%) patients were downstaged to N1, 27(33.3%) patients remained N2 ,while 2(2.4%) patients progressed to N3. Of N3 patients (N=10), 9(90%) patients were downstaged, 6(60%) to N2 and 3(30%) to N1, while 1(10%) patient remained N3. This downstaging in the axillary lymph node status was found to be statistically significant ($p < 0.05$).

The clinical response however did not have any significant correlation with the pre NACT tumor size, age and menopausal status of the patients.

In our study 56 (36.8%) patients were in low Hb group, while 96 (63.1%) patients were in normal Hb group. It was observed that of 56 anemic patients 38 patients (67.8%) were nonresponders and 18 (32.1%) were responders, while of 96 nonanemic patients, 87 (90.6%) patients were responders and 9 (9.3%) were nonresponders. Thus a statistically significant correlation was observed between anemia and poor clinical response.($p<0.05$)

In the biopsy specimen before initiation of NACT , 57 (37.5%) patients were p53 +ve and 95 (62.5%) were –ve. Immunohistochemistry was also done for Bcl-2 and VEGF and it was observed that 52(34.2%) patients were Bcl-2 positive and 54(32.8%) patients were VEGF positive.

Tumor suppressor gene P53, anemia and clinical response

It was observed that of 57 p53 +ve patients 41(71.9%) were nonresponders and 16(28%) were responders. While of 95 p53 –ve patients 89(93.6)% patients showed response and 6(6.4%) were nonresponders. And this correlation between p53 positivity and poor clinical response was statistically significant.($p<0.05$)

Of 56 anemic patients, 45(80.3%) were p53 +ve and 11(19.6%) were –ve. While of 96 nonanemic patients only 12 (12.5%) were p53 +ve.Thus a statistically significant correlation was observed between low Hb level and high p53 expression.($p<0.05$)

It was also observed that of 45 p53 +ve anemic patients 36(80%) were nonresponders and 9(20%) were responders. Thus a significant correlation was observed between anemia, p53 positivity and poor response.($p<0.05$)

BCL-2, anemia and clinical response

Of 52 Bcl-2 +ve patients only 9(17.3%) were responders and 43(82.6%) were nonresponders. While of 100 Bcl-2 –ve patients 96 (96%) were responders and 4(4%) were nonresponders. This correlation between positive Bcl-2 expression and poor clinical response was statistically significant.($p<0.05$)

Of 56 anemic patients, 42(75%) were Bcl-2 positive and 14(25%)were Bcl-2 negative. While of 96 nonanemic patients only 10(10.4%) were Bcl-2 +ve. Thus a statistically significant correlation was observed between low Hb level and high Bcl-2 expression.($p<0.05$)

It was observed that of 42 Bcl-2 +ve anemic patients, 37 (88%) were nonresponders and 5(11.9%) were responders. Thus a significant correlation was observed between anemia, Bcl-2 positivity and poor response.($p<0.05$)

VEGF, anemia and clinical response

Of 54 VEGF +ve patients, 45 (83.3%) were nonresponders and 9 (16.6%)were responders, while of 98 VEGF –ve patients, 2(2%) patients were nonresponders and 96 (97.9%) were responders. This correlation between positive VEGF expression and poor clinical response was statistically significant.($p<0.05$)

Of 56 anemic patients 44(78.6%) were VEGF +ve and 12 (21.4%) were VEGF –ve. While of 96 nonanemic patients 10(10.4%) were VEGF +ve. Thus a statistically significant correlation was observed between low Hb level and high VEGF expression.($p<0.05$)

It was observed that of 44 VEGF +ve anemic patients, 39(88.6%) were nonresponders and 5(11.3%) were responders. Thus a significant correlation was observed between anemia, VEGF positivity and poor response.($p<0.05$)

Discussion

Breast cancer is the commonest malignancy in women worldwide and more than 1,000,000 new cases are diagnosed every year[20,21]. Although the incidence has increased over last 20 years, the prognosis has improved, partly because of early diagnosis and as a result of more active treatment against systemic spread[21].

Carcinoma of breast is a leading cause of cancer mortality in women all over the world and the second most common malignancy in India after carcinoma of the uterine cervix[1,23-26]. In India like in other developing countries 25–30% cases are locally advanced at the time of diagnosis[1,23-26]. The recommended approach for the management of LABC is a multimodality approach intended to provide both local and systemic control and studies have confirmed that surgery alone is an inadequate treatment[23,26]. The realization that patients with LABC are likely to have undetectable micro metastases at diagnosis has lead to systemic treatment assuming an important role, as even aggressive surgical techniques do not reduce the higher incidence of local recurrence. Most importantly surgery does not change the pattern of distant failure in these patients as they often have micrometastatic disease at the time of diagnosis[23].

Neoadjuvant chemotherapy was first introduced with a 70% objective response rate in 1970s and was initially utilized to convert unresectable tumors to smaller tumors making them more amenable to local control with either surgery or radiotherapy. Although the correlation between the tumor response and prognosis is still uncertain, it is generally believed that such a relationship may exist[23,25]. The other important advantage of NACT is

that it provides an *in vivo* chemosensitivity test for assessment of tumor response from which prognostic information could be obtained.

Apoptosis

Normal breast development is controlled by a balance between cell proliferation and apoptosis, and there is strong evidence that tumour growth is not just a result of uncontrolled proliferation but also of reduced apoptosis[20].

Apoptosis is a closely regulated form of active cell death defined by characteristic biochemical and morphological criteria. A large number of anti-cancer agents with widely differing modes of action have been demonstrated to induce apoptosis in vitro, suggesting this as a significant final common pathway for exerting their clinical effects. Mechanisms that suppress apoptosis may be important in the development of intrinsic and acquired resistance to cytotoxic drugs[1,4].

Apoptosis is a regulated phenomenon capable of being inhibited and activated. Indeed there is evidence that stimulation of some cells by trophic cytokines or increase in their levels of expression of Bcl-2 proto-oncogeny can greatly increase their resistance to the apoptosis-inducing effects of anticancer drugs. Thus Bcl-2 proto-oncogeny expression may be implicated in the development of resistance of tumors to therapeutic agents and may contribute to tumor growth and perhaps to oncogenesis by allowing the inappropriate survival of cells with DNA abnormalities[1,27].

Deregulated expression of the Bcl-2 protein represents the best-known example of a potent blocker of apoptosis. Over expression of Bcl-2 has now been shown to protect a wide variety of cell types from induction of apoptosis by many different anticancer agents[1,4].

The other marker which is studied extensively in breast cancer is tumor suppressor gene ,p53. The protein product of p53 controls cellular functions involved in apoptosis, the cell cycle, and the repair of DNA.[20,28] Mutations in this gene are the most common mutational event in cancer. Mutations in the gene or increased expression of the p53 protein (an indirect marker of mutation as this often results in stabilisation of the protein), has been associated with a poor prognosis to breast cancer in some studies[20,29]. Those studies that have measured mutation as opposed to protein over expression have consistently shown that mutated p53 is related to a poor response to chemotherapy.[20,30]

Anemia and tumor hypoxia

Anemia is a major problem worldwide , more so in developing countries[7]. The prognostic impact of anemia in cervical cancers is well established. In a recent meta-analysis, anemia was found to be an independent prognostic factor for increased death rates also in various solid tumors(head and neck, lung, prostate) as well as lymphoma[7]. It is been seen in studies that there is not a linear relationship between Hb level and local failure rates or survival[7]. Hb levels in the normal or nearly normal range do not correlate with failure rate[7]. Evans and Bergsjo have demonstrated that patients with Hb level of less than 11 gm/dl had a significantly poorer overall survival as compared with patients with Hb level of 11g/dl or more.[7,33] The relation between low Hb levels and a low oxygenation status of malignanttissues could be demonstrated in several studies[10-14]. Tumor hypoxia is a direct consequence of structural abnormalities of the microvasculature and functional impairments of the microcirculation and results from either limited pO2 diffusion (chronic hypoxia) or limited perfusion (acute hypoxia)[11]. Studies suggest that tumors respond more sensitively to a lower Hb concentration than normal tissues. A possible explanation for this phenomenon could be that the inadequate vascular structure in malignant tissue is not able to compensate for the deficiency of oxygen carriers with a decreased vascular resistance and enhanced flow[11].

Effect of tumor hypoxia on apoptosis

Poor oxygenation of residual and accelerated repopulating tumor cells may have severe effects on tumor cell biology. Hypoxia is one of the reason for genetic instability and

the development of mutations in malignant tissues[10,17,18]. Such mutations could affect genes encoding for apoptotic cell death. An example of such a gene is p53 tumor suppressor gene, which induces apoptosis in hypoxic tumor cells. Genetic alterations promoted by hypoxia could result in a loss of functional p53 tumor suppressor gene and therefore in a loss of apoptotic potential in hypoxic tumor cells[10,19]. In vitro studies demonstrated that repeated hypoxic exposure resulted in overgrowth of mutant p53 clone i.e decrease in functional p53.[31]

Another form of of genetic alteration in hypoxic tumor cells is overexpression of the apoptosis inhibitor protein Bcl-2. According to Graeber et al. an overexpression of this protein could be demonstrated especially in hypoxic tumor cells[10,19].

Angiogenesis

One very important prognostic indicator which is widely studied for breast cancer is angiogenesis. Angiogenesis is necessary for the growth and invasiveness of primary tumors and is integral part of cascade of biologic events involved in tumor metastasis[21]. The mechanisms by which neovascularisation stimulates tumor progression are as follows:

1) delivery of nutrients and oxygen necessary for tumor cells to grow(perfusion effect).

2) facilitation of penetration of tumor cells through vessel wall and their transport to distant organs(metastatic effect).

3) secretion of some cytokines (IL 1-6 and 8) and growth factors(G-CSF, TGF-beta-1, IGF and angiogenic peptides) from endothelial cells that directly stimulate tumor cells(paracrine effect). The switch to angiogenic phenotype may be due to the overexpression of number of endothelial growth factors and/or to reduced expression of endogenous angiogenesis inhibitors[21].

Vascular endothelial growth factor, also known as vascular permeability factor, is a potent and widely distributed angiogenic peptide.[21] VEGF is a heparin-binding glycoprotein that has several important effects on vascular endothelial cells. Currently VEGF is considered to be most selective mitogen for endothelial cells.[32] VEGFs are polyfunctional molecules that have been implicated in vasculogenesis, endothelial cell proliferation and migration, vascular permeability and stromal degradation through activation of some proteolytic enzymes involved in tumor invasiveness and angiogenesis.[21] VEGF expression is increased in response to various stimuli, including certain oncogene products e.g mutant ras[21]; and overexpression of transforming growth factor- alpha[21]; and hypoxia[21].

The most stimulating factor for release of VEGF is hypoxia in surrounding tumor cells[10,17]. In consequence, hypoxic malignant tissue could more rapidly develop a sufficient vascularisation than normoxic tumors. For microscopic residual cells under hypoxic conditions this means that they attain a sufficient blood supply by the release of VEGF much faster than normoxic residual tumor cells. A relapse of disease and regrowth of tumor tissue therefore is more likely in patients with low Hb levels[10,17].

There seems to exists a threshold in the range of about 11 g/dl below which the prognosis dramatically worsens[7]. the presence of such a threshold has also been demonstrated by by Evans and Bergsjo[7,33]. Based on these observations we divided the patients in two groubs taking Hb level 11 g/dl as the cut off value.

In our study we could not find any relation between clinical response and patient age, pre NACT tumor size, menopausal status.

In this prospective clinical study it was observed that patients of LABC with pretreatment low Hb level have poor clinical response to NACT, while nonanemic patients have good response to NACT. 67.8% of anemic patients were nonresponders, while 90.6% of nonanemic patients were responders to NACT.

In our study it was observed that anemic patients have increased expression of mutant tumor suppressor gene p53 (i.e nonfunctional p53), Bcl-2 and VEGF. Increased expression

of p53 and Bcl-2 lead to decreased apoptotic cell death (the mechanism responsible for chemotherapy induced tumor cell death) and thus chemoresistance. Overexpression of VEGF leads to increased angiogenesis and thus increased tumor invasiveness. This increase in angiogenesis also leads to chemoresistance.

Correction of Hb level before NACT can avoid chemoresistance. Correction can be done with blood transfusions and erythropoietin along with hematinics[8].

Conclusions

This study highlights the importance of hemoglobin level in predicting response to NACT in breast cancer patients. While many biological markers are in use and many are under trial to tailor the chemotherapy for a particular patient, most of these markers including apoptotic markers or p-glycoprotein etc. are not very frequently available and are expensive for a third world cancer set up. Patients with low Hb level before initiation of NACT were found to be poor clinical responders. Pretreatment low Hb level may thus be utilized as a predictor of response to NACT and thus correction of Hb should be done before initiation of treatment in order to avoid chemoresistance.

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