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Biomarkers That Predict The Response to Chemo-radiotherapy in Rectal Cancer

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ABSTRACT

The treatment of locally advance rectal cancer is a challenging with a relatively high incidence of local recurrence. CRT, however, has proven to be a great success in controlling rectal tumor locally. Nevertheless, recognizing relevant biomarkers are of paramount importance in order to tailor patient management and to avoid unnecessary chemotherapy related morbidity. In addition, biomarkers could outline oncological outcomes early on. Tumor response considered a strong indicator about tumor status and response to the given treatment. Unfortunately, there is no single effective method to predict tumor response to CRT treatment solely. However development of clinical and immunohistochemistry markers are essential to know what it takes to design a proper management plan for the right patient. We focus in our review to summarize relevant articles emphasizing predictive value of tumor biomarker in estimating rectal cancer response to preoperative CRT.

INTRODUCTION

Rectal cancer accounts for third common cancer related cause of death in females and second in males in South Korea. Since the introduction of national screening program, early stage rectal cancer has steadily risen. Despite successful initiation of screening program, locally advance rectal cancer still accounts for considerable volume, reaching 40%. Preoperative chemo-radiotherapy (CRT) was recommended in locally advanced rectal cancer to control local recurrence compared with post-operative CRT but with similar overall survival (1-6). Advert of CRT in rectal cancer has been attributed to achieve complete pathologic response (pCR), which correlates with improved survival, decreased local recurrence, and a higher rate of sphincter-preserving surgeries (7-9). On top of that, numerous studies have proposed to estimate two important parameters to assess advanced rectal tumor; the tumor response to chemo-radiotherapy (CRT) and the relation of patient oncological outcome to a given treatment. If we do so, then we probably could interfere at the right time with proper target agents in order to mitigate tumor burden and as a result improve patients quality of life.

Monitoring the tumor response particularly during CRT treatment in terms of clinical and radiological response is essential in order to maximize patient care. There are several ways to estimate tumor response which would then guide surgical management accordingly. However, there is no single parameter designed to assess tumor response solely, thus warranting multidisciplinary approach. Tumor biomarkers have dramatically evolved in the field of the colorectal cancer. Its role has contributed in the treatment strategy. Nevertheless, conflicting results among published articles have made decision making is uncertain and complicated.

Supportive evidence for the true role of tumor biomarker in CRC is lacking. Several randomized studies are investigating these marker in order to boost treatment options and to predict for those with poor predictive measures. Due to the necessity of predictive biomarkers in the field of colorectal cancer, we focus our review to discuss the value of tumor biomarkers to predict tumor response after preoperative CRT and its impact on tumor prognosis and overall survival.

Clinical Value in Assessing Tumor Biology

Tumor biology and aggressiveness varies among individuals according to patient factors including age and sex and familial disease background as well as tumor

factors such as tumor size, location and level of tumor depth and whether they invade adjacent structures or not. Overall, nature of the tumors have a very wide range of prognosis, from low-grade tumor with high responsiveness to chemotherapy and high-grade tumor with dismal oncological outcomes. To perform extensive surgery for locally advance tumor is a challenging decision. Therefore, prediction of tumor behavior is essential in order to set management plan and it may avoid extensive surgery in a certain aggressive tumor background. We aim to address our experience along with other well-designed trial posted in this regards in the following section.

Laboratory Evaluation

Complete blood counts, liver function tests, coagulation profiles and various other laboratory tests are routinely performed during evaluation and over follow up period. None so far have demonstrated an accurate role in assessing the response to neo adjuvant treatment. However one or more of the following may play a role in evaluation of the response to neoadjuvant CRT.

Carcino-embryonic Antigen

Serum carcinoembryonic antigen (CEA) is most widely used tumor marker in patients with CRC. Its role for prediction of prognosis and follow up of patients for recurrence has already been established [10]. Improved survival has been shown with reduction of the CEA level after radical resection [11]. We review the data for CEA levels before and after neoadjuvant CRT in determining complete *vs*. incomplete responders. Meta analysis performed by Huichuan Yu et al, who included 3,705 cases found normal CEA (<5 ng/ml) predicted improved pathological complete response (pCR) (P<0.00001) and good response (P < 0.00001) to CRT [12]. Das P et al performed retrospective review of 562 patients who received preoperative CRT and found CEA level >2.5 ng/mL were associated significantly with a lower pCR rate compared to low CEA level <2.5ng/ml, rated at (11% vs. 24%, p=0.15), respectively.[13]. Zeng et al in their retrospective study found that 76.0% of the patients with a pCR had a normal pretreatment CEA level, versus 58.5% of the patients in the non-pCR group [14].

Various studies have used different cut off point for CEA level. Meta analysis [12] used 5 ng/ml as cut off while 2.5ng/ml [13,15], 3 ng/ml [16] or 3.5 ng/ml [17] have been used as cut off in other reports. All of this data were also found to be of significant value for prediction of pCR. Overall, similar studies showed that normal CEA level is correlated with improved pathologic response to neoadjuvant therapy, while high CEA level may predict a poor response.

Nevertheless, most of the studies included were retrospective, and few subset studies had small number of cases, which decreased the reliability of the results. Also there is a significant variability in terms of cut-off values of CEA levels among studies, which made it difficult to acquire strong evidence to conclude. Despite the limitations, the current studies including the meta-analysis suggests that pretreatment normal CEA levels is a useful predictive factor for response to neoadjuvant chemoradiotherapy in patients with rectal cancer.

Time Interval Between CRT and Surgery

Data suggests that there is a definite association between the time interval from CRT completion to surgery and tumor response rates with pCR. Literally, the principles of delaying surgery after radiotherapy is to give sufficient time for cell destruction after radiation. DNA damage post radiotherapy occurs during irradiation whereas cellular lysis occurs within next few weeks. Therefore, several retrospective studies are in lines with this treatment plan.

In 1999, Lyon R90-01 trials [18] compared 2 weeks vs. 6 weeks delay of surgery post CRT and demonstrated that a delay up to 6 to 8 weeks increased the clinical tumor response and pathological downstaging. Recently, one of the largest meta-analysis of 17,255 patients published by Probst et al [19], showed that an interval time >8 weeks was associated with more odds of pCR without any evidence of increased surgical complications as compared with an interval of 6 to 8 weeks. Another meta analysis of 3584 patients by Petrelli et al [20] showed similar results. It suggested that a longer waiting interval of more than the 6–8 weeks from the end of preoperative CRT had pathological complete response increased from 13.7% to 19.5% in the longer interval group that is almost 6% rise, whereas the overall survival, disease free survival, R0 resection rates, sphincter preservation, and complication rates were similar in both the groups.

Despite that, the actual duration of ideal waiting period is still unknown. Considering this, GRECCAR6 trial [21], phase III, multicenter, randomized, open-label, parallel-group controlled trial was initiated. The aim was to evaluate the effect of a longer interval that is 7 weeks vs. 11 weeks between the end of CRT and surgery on the pCR rate in a randomized trial. It showed that waiting for 11 weeks interval was not associated with an increase in the pCR rate but the delay was associated with a higher postoperative morbidity rate as a result of increased medical complications and a worse quality of mesorectal excision probably due to difficult pelvic dissection and/or a more fragile mesorectum because of the longer interval. Thus, from our point of you, we follow a strategy of 6-8 weeks interval after CRT then reassessment of the tumor response a week before planning the surgery is justified to design any further evaluation.

Endoscopic Findings:

Second important parameter to consider is endoscopic evaluation. Indeed, it accounted for the only way possible to grossly identify the tumor response after CRT. Various studies have been under taken to identify tumor response on endoscopy. Habr-Gama, et al [22] in 2010 proposed the following findings suggestive of complete response post CRT as could be identified on endoscopy.Whitening of the mucosa, telangiectasia, signs of harboring the scar and no residual tumor. On the above findings they suggested that Wait and Watch Policy with regular assessments could be a safe option. Ogura A, et al [23] in 2015 published their findings comparing E-CR (Endoscopic Complete Response)

to pathological complete response and E-non CR (Endoscopic non complete response) to Pathologic Non complete response. Flat scar, disappearance of the neoplastic pattern and disappearance of the neoplastic nodule or stenosis are suggestive of E-CR. They concluded that evaluation of E-CR along with histopathologic response showed an accuracy of 91.7 %, sensitivity of 27.8 %, specificity of100%, positive predictive value (PPV) of 100 %, and negative predictive value (NPV) of 91.4 % ($p<0.001$).

From our personal experience, we are in line with these results. We (kim et al) investigated 71 patients diagnosed with rectal cancer after CRT(24). We defined cCR by endoscopic finding as follow 1) No visualization of tumor, 2)White scar, or red scar were included in "cCR" 1) ulcerations and 2) remaining masses of any size were considered "non-cCR." The results showed 24(33.8%) patients had pCR. 23 patients had demonstrated E-CR of which 19 (82.6) patients showed pCR. 48 patients demonstrated endoscopic non-cCR of which 43 (89.6%) showed non-pCR. We concluded that endoscopic findings had exhibited 81.8% sensitivity and 91.8% specificity.

Thus endoscopy plays a significant role to identify tumor response after CRT. The only caveat is that it only gives us information of the luminal side and that it does not provide information regarding mesenteric and nodal response. In spite of that, endoscopy provides high specificity for assessment of response as shown by the above mentioned two studies and it is thus a valuable predictive tool.

Radiological Assessment of Tumor Responsiveness after CRT

Magnetic Resonance Image (MRI) Scan

MRI plays an important role in the staging of rectal cancer but its role in assessing patients with pre operative CRT is still in doubt. This is mainly due to both overstaging of T1 or T2 tumors which occurs due to radiation induce fibrosis and inflammation along with radiation induce ulceration and proctitis and understaging which is mainly due to inability at times to detect small underlying tumors which are covered by fibrosis[25]. MRI criteria to identify nodes typically depends on its size that is short axis measuring above 0.5cm [24]. Post CRT usually there is alteration in lymph node structure caused by fibrosis, which may increase the false positive rate[25]. MERRION trail[26] stated similar conclusion suggesting that MRI would understage one third of node positive tumors and overstage one third of node negative tumors. One of the trials in favor of MRI post CRT is the MERCURY group[27]. They proposed that a trained radiologist could differentiate fibrosis from tumor on MRI scans as fibrosis has very low signal intensity compared to tumor that has intermediate signal intensity. However they also mentioned that negative predictive value for circumferential resection margin (CRM) was 98% and specificity for CRM was 73%. Thus, there would be less chances of involved margins and over treating outside total mesorectal excision (TME) plane[27]. A meta analysis of pooled date by Paardt et al[28] on more than 1500 patients showed a low sensitivity of 50% for assessment of ypT stage and on the other hand it showed relatively high specificity of 91%. They also showed that for ypT0 sensitivity was only 19% with a high specificity of 94%. They concluded that fair results were observed with MRI for restaging CRM but nodal staging remained doubtful. The above data suggest limited role of MRI in detection of response following CRT.

Recently the advances in functional magnetic resonance technology combined with morphological information could serve as imaging biomarkers providing quantitative data of the tumor. Functional MRI thus could provide a better picture of tumor heterogeneity along with changes on response to treatment. There are reports that show that combination of visual assessment of tumor along with objective assessment of tumor shrinkage by MRI volumetric measurements can give results of accuracy as high as 87%[29,30]. Dresen, et al showed accuracy of 87% to predict yp T0-2 when combined with initial tumor volume of <50% and a>75% volume reduction rate post CRT were used together.

In a study evaluated by our institute, prospectively we evaluated the impact of tumor volume changes by 3D volumetry on tumor response in 84 patients with post CRT followed by radical surgery wherein 3D MR volumetry were compared with histopathological response. The results in multivariate analysis showed the tumor volume reduction ratio was not significantly associated with T or N down staging, although the volume reduction ratio(>75%, $p=0.01$)was significantly associated with an increased pCR[31].

Emerging new functional MRI with two version of which are currently in use are perfusion imaging also known as dynamic contrast enhanced MRI (DCE-MRI) which demonstrates underlying tissue cellular architecture and the other being diffusion weighted MRI (DW-MRI), which demonstrates angiogenic vascular activity of the tumor. Various studies have shown the effect of MRI and DWI imaging improving the accuracy in detecting the tumor post CRT[28,32,24]. Meta analysis by Paardt et al[28] showed the rate of detection increased by 84% with minimal risk of missing the tumor on addition of DW imaging. An article published in our institute by kim et al[24] mentions the importance of both DCE-MRI and DW-MRI and concludes that DW-MRI is better imaging biomarker than DCE-MRI post CRT.

Recent advances in MRI are Magnetic resonance spectroscopy (MRS) specially using proton (H) as metabolite and others being image segmentation using histogram analysis and texture analysis which are still under investigation[33]. Considering a more readily availability of functional MRI techniques as of the diffusion or perfusion MRI the role of the newer techniques is still in question. Further studies are required regarding their use in detection of response post CRT.

To summarize regarding MRI, the decision should be individualized at multidisciplinary team (MDT) meetings as there are no clear guidelines across the board, however MRI seems to have high application in the field of tumor assessment due to its affordable cost and low radiation toxicity profiles.

Positron Emission Tomography (PET) Scan

18-fluoro-2'-deoxy-*D*-glucose (FDG-PET) is a well-known modality in diagnosis of various cancers. It works on the principle that tumor cells have enhanced glycolysis and this cellular glucose metabolism separates them from surrounding normal metabolic active tissues.
The advantage of it being non invasive and its reasonable availability along with its ability to detect metabolic activity in residual tumor cells post CRT in rectal cancer makes it one of the investigations to consider[34].There are various things to consider with regards to PET. Firstly, there is debate regarding time interval between end of CRT and the timing of PET.
It is well known that CRT causes fibrosis and inflammation and this results in accumulation of FDG uptake, which may last few weeks to months. Moreover, CRT causes "stunning" effect, which is reversible temporary effect, in which there is decreased glucose metabolism in viable tumor cells, which may last for weeks. Findlay, et al [35] evaluated patients 1 to 2 weeks post treatment and again 4 to 5 weeks later and they found that scan 4 to 5 weeks post treatment-identified responders from non-responders with 100% sensitivity and 90 % specificity.

Kristiansen, et al [36] performed scans at 7 weeks and found the sensitivity and specificity was 44% and 64% respectively and positive predictive value and negative predictive value were 58% and 50% respectively. Whereas Jassen, et al [37] demonstrated that scan on day 15 post CRT was the best predictive factor for response post CRT. Similarly Cascini, et al[38] compared early(14- 17 days post CRT) to (8 weeks post CRT) and found that early PET scans were better predictors of response with overall accuracy of 94% and sensitivity and specificity of 100% and 87% respectively.

Secondly, it is not clear which parameters would predict better response compared to others.
Various parameters have been studied, most commonly being SUVmax (Maximum Voxel standardized uptake value), which is a ratio of radioactivity concentration to the injected activity divided by body weight, ΔSUV which is the difference between pre SUVmax and post SUV max, Response index(RI), which is (RI)=[ΔSUV/pre-SUV]×100, ΔTLG% which is defined as percentage change in total lesion glycolysis (TLG) before CRT and after CRT. Visual Response (VR) was one of the qualitative parameters assessed. Maffione, et al[39] studied 8 parameters and suggested that SUV max, VR, RI and ΔTLG% corresponded more with pCR of which SUV max-post had the highest sensitivity. Amthauer et al[40]. suggested that ΔSUV was higher in responders than non responders with sensitivity and specificity of 93% and 100% respectively. Also, Capirci, et al [41] concluded that RI was the best predictor of response with sensitivity and specificity of 84.5% and 80% respectively.

From our experience, we found out that SUV-post and RI were significantly related to pCR with sensitivity and specificity of 84.6% and 79.2%, respectively[42]. Point to consider is that all these different studies used different cutoff point for each parameter thus causing different diagnostic accuracy. Overall various studies irrespective of the criteria used and the cut off value considered have found sensitivity and specificity of PET to be ranging between 45%-85% and 79%-81%[25].Another drawback of PET is its weakness to identify lymph node metastasis and in its inability to differentiate between residual tumor and inflammation or fibrotic changes post CRT[24]. Despite of all the drawbacks a meta-analysis by Zhang, et al[43] concluded that PET is valuable to predict response post CRT and it is more promising when used early during treatment and they found no difference between RI, SUV and VR values in predicting response.

With a view to improve efficacies various studies have combined PET with other modalities to determine response post CRT. Metser, et al[44] in their study suggested that parametric fusion PET-MR may improve prediction of response post CRT. In summary PET alone is far from being an ideal marker to predict response post CRT. For better prediction it is necessary to utilize functional imaging (18F-FDG PET) with morphological imaging techniques (TRUS, MRI) for best possible outcomes.

Application of Molecular Biomarkers in the Tumor Response

In spite of all the available radiological and endoscopic modalities and attempts to standardize findings for the same, the assessment is rather subjective and operator dependent. Even post preoperative treatment biopsy is inconclusive at times. Therefore, evaluating tumor response based on subjective measures are of paramount important in order to tailor management plan individually. CEA has solely been recognized as one of the most largely used markers in colorectal surgery. But as mentioned its use as a sole marker for prediction of response is debatable. Therein, various molecular biomarkers have been investigated in order to search for an appropriate assessment tool of tumor response post CRT in rectal cancer using immunohistochemistry (IHC) or direct gene sequencing analysis. There are numerous markers approved and there are others still under investigation, however we will focus on our report on the well-studied biomarkers and their controversies with regards to their role in tumor responsiveness after CRT.

Tumor Suppressor Genes

p53 Gene

Tumor suppressor p53 protein is a stress-inducible transcription factor, function of which is to regulate large number of diverse downstream genes and thus exert regulative function in multiple signaling process leading to induction of cell cycle arrest, senescence, and apoptosis under cellular stress response and inhibition of angiogenesis.
Carcinogenesis of CRC is multistage and multifactorial involving the activation of oncogenes and inactivation of tumor suppressor genes. Approximately 40% - 50% of sporadic CRC have p53 mutation[45].CRC progression usually follows mutations of APC, K-Ras, and p53 genes[46]. p53 is considered to play a vital role in the adenoma-carcinoma sequence for development of CRC[47]. P53 mutation reported in 34% of proximal colon tumors and 45% of distal colorectal tumors. [48].

Different types of p53 mutations play different pivotal roles in determining the biological behavior of CRC as in invasive depth, metastatic site and prognosis. In proximal colon cancers p53 mutation are associated with lymphatic invasion whereas in distal CRC it is associated with both lymphatic and vascular invasion[48]. It is already established that wild-type p53 mutation in malignant cells is correlated with sensitivity to radiation or chemotherapy induced damage whereas mutated p53 is correlated with radio and chemo-resistance[49].

Many studies have been undertaken to show the correlation between p53 mutation and response in CRC. There have been contrasting results. Komuro et al.[50] and Fu CG, et al.[51] in their studies showed that detection of nuclear p53 using IHC is associated with resistance to pre operative CRT. Similarly study by Spitz, et al.[52] showed an inverse relation between presence of p53 staining and complete histopathologic response and direct relation between p53 staining and residual tumors in lymph nodes detection post surgery. Lin, et al.[53] suggested that p53 negative tumor biopsy before radiation would respond better to radiation and induce apoptosis compared to p53 positive cells, thus indicating better response in p53 negative cells.

Meanwhile studies by Bertolini, et al.[54] and Chen, et al.[55] suggested no correlation between p53 mutation and tumor response. On the other hand Esposito, et al. [56] found contrasting results suggesting that positive pretreatment tumor biopsy for p53 showed better response to preoperative CRT.

From our recent study conducted by Hur et al [57], we examined clinical impact of different biomarkers on tumor responsiveness in 81 patients with locally advanced rectal cancer. Following 12 biomarkers were included; p53, p21, Bcl2, Bax, EGFR, Cox-2, MLH-1, MSH-2, Ku70, VEGF, TS, Ki-67. Patients showing low expression of p53 and/or high expression of VEGF, p21, and Ki67 exhibited a significantly greater pCR rate after CRT. Thus, we are in line with those showing strong correlation between p53 mutation positivity and tumor resistance to CRT.

p21 Gene

DNA damage in cells activates p53 – p21 pathway, which induces senescence in cells. Senescence is a form of cell cycle arrest which is prolonged and irreversible. The purpose is to provide cells enough time to repair the damaged DNA and to avoid accumulation of harmful mutations in the daughter cells thus preventing development of potential cancerous cells[58].There are various stress stimuli in the form of DNA damage response, oncogenes, oxidative stress, dysfunctional telomeres, all of which usually trigger p53 – p21 pathways to induce senescence[59].

Presence of wild type form of tumor suppressor p21 in cancer cells induces apoptosis in response to DNA damage caused by radiation and chemotherapy drugs. There is data suggesting that loss of wild type p21 or presence of mutant p21 sensitizes tumor cells to radiation[57]. As with p53, studies conducted with p21 shows various results. Bertolini, et al.[54] in their study showed no correlation between p21 expression and response to CRT although they did show reduction in disease free survival with high p21 expression. Whereas Reerink , et al.[60] suggested that patients with higher p21 expression were associated with poorer survival. On the other hand Rau, et al.[61] and Charara, et al.[62] in their respective studies suggested that p21 expression was associated with pathological complete response. From our previous report we found similar results with Rau and Charara suggesting that p21 expression was significantly associated in good responders and was associated with pathological complete response[57].

Growth Factors Proteins

Vascular endothelial growth Factor (VEGF)

Angiogenesis is one of the most important factor for tumor growth and metastasis as shown by in vivo studies[63]. Angiogenesis possess greater risk for hematogenous tumor embolization. Thus theoretically inhibiting angiogenesis may halt tumor growth and decrease chances of metastases. Most commonly associated factor with angiogenesis is vascular endothelial growth factor (VEGF). Along with its ability to induce angiogenesis it has ability to induce vascular permeability, which allows diffusion of proteins into interstitial space and provide a framework on which endothelial cells migrate [63]. In addition, VEGF is one of the hypoxia markers, which has been studied in response to rectal cancer.[57]. Hypoxia causes resistance to radiation and chemotherapy by depriving cells of oxygen, that is essential for cytotoxic activity of these agents.

Many studies have implicated the role of VEGF in CRC angiogenesis, metastasis and proliferation of cancer [64]. Blocking VEGF receptors results in normalization of vasculature and vascular permeability which in turn decreases interstitial pressure thus increasing oxygen transport to cells, which potentiates effect of radiotherapy and also helps deliver chemotherapeutic agents to target therapy[65]. Study by Zlobec, et al.[65] showed that strong VEGF positivity was associated with it being more radio resistant, thus suggesting that VEGF negative tumors should be good candidates for pre op CRT. Another study by Giralt.Et al.[66] suggested similar results stating VEGF positivity to be associated with poor disease free survival following CRT. On the other hand, Qui, et al.[67] demonstrated no relation between VEGF expression and tumor response post nCRT.

In contrast, our study observed that VEGF expression was associated with pCR and those tumors that strongly expressed VEGF on Tissue Microarray (TMA) responded better to CRT than those with weak expression[57].Therefore, VEGF is a very important subject to be addressed in the future as its direct relation for development of monoclonal antibody and target agent could improve tumor control and improve oncological outcome.

In a study from our institute in 350 CRC cases by Jang et al [68], we genotyped 4 polymorphisms of VEGF namely -2578C>A, -1154G>A, -634G>C, and 936C>T using polymerase chain reaction-restriction fragment length

polymorphism assay. We found that VEGF -2578CA genotype was significantly associated with poor prognosis for rectal cancers and has poor overall survival in all patients enrolled. Thus, VEGF has a potential to be a genetic marker. Table 1 illustrates correlation between tumor response and molecular biomarker.

Epidermal Growth Factor Receptor (EGFR)

Epidermal growth factor receptor (EGFR) belongs to a family of receptors known as the ErbB family (ErbB tyrosine kinase receptors). They comprise of four proteins that are encoded by c-erb B proto-oncogene: EGFR itself or ErbB1, ErbB2 or HER2/neu, ErbB3 or HER3 and ErbB4 or HER4. The function is to activate multiple signaling pathways that facilitate tumor growth process. Various studies have shown EGFR overexpressed in CRC but its value, as a prognostic marker is still unclear. It is also reported to be correlated with more aggressive disease, advance stage of disease, metastasis, and lymph node involvement[69].

Very few studies have evaluated the predictive value of pretreatment EGFR in CRT. Giralt, et al.[70] suggested that patients with EGFR- negative tumor were significantly associated with pCR than EGFR –positive patients. Bertolini, et al.[54] found no correlation between EGFR and response to nCRT. Zlobec I, et al.[65] suggested that pretreatment EGFR positive patients had better chances of complete pathological response and they should be treated with nCRT. Kim, et al.[71] in their multivariate analysis demonstrated that low EGFR expression significantly predicted tumor down staging post nCRT.

Other Molecular Biomarkers

Ki67

The growth and proliferation of tumor cells is highly variable and this reflects their clinical course, although proliferation is a key feature of tumor growth. Ki67 is a nuclear antigen seen in proliferating cells and is detected in all active stages from G1 to M-phase of cell cycle.
When Ki67 has been blocked by either blocking antibodies or by inhibition of dephosphorylation there has been arrest of cell proliferation. Ki67 staining is measured as Ki67 index, which is the number of Ki67 staining tumor cells per 1000 cells. The correlation of Ki67 index with response in CRC is still unclear. Jakob, et al.[72] in their study concluded that patients with significantly lower Ki67 index had better response to CRT.
In Our institute, two studies were conducted to assess correlation between Ki67 and response post CRT. First study showed significant positive correlation between cellular proliferation index and tumor response in rectal cancer after CRT [73]. Whereas in a second study conducted by our institute we found that Ki67 index was significantly higher in patients who responded well to CRT based on ypTNM, T down staging, N down staging, TGR, and pCR[57]. It was also noted that Ki67 was independent predictor of pCR. Based on this data, Ki67 is a predictor however, results are conflicting with debates still remained.

Cyclooxygenase 2 (COX2)

COX2 is a known mediator of tumor invasiveness and metastasis. COX2 overexpression is found in approximately 50% of adenomas and 85% of adenocarcinomas[74]. Davis T, et al.[75] suggested that addition of selective COX2 inhibitors in patients can significantly increase response to radiation by reducing prostaglandin release. Shinto, et al.[76] in their both univariate and multivariate analysis demonstrated strong correlation between COX 2 expression and resistance to CRT. Smith, et al.[77] showed a similar result in their study suggesting that patients with COX 2 overexpression receiving CRT were more likely to show poor response as compared to those with normal COX 2 expression.

In a study conducted by our institute the results were similar suggesting that patients with COX 2 overexpression were more likely to respond poorly to nCRT and less likely to achieve histopathological nodal down staging as compared to those with normal expression[78].

BCL-2 / BAX

Correlation of BCL -2 expression and various cancers especially solid tumors like prostate, lung and colon cancers is well known[79]. BCL-2 and BAX (which is a subset of protein from BCL-2 family) belong to oncogenes involved in the apoptosis pathway. BCL-2 functions to block the induction of apoptosis by anticancer drugs. There are debates regarding BCL-2 expression and its correlation to chemotherapeutic drug, as at times overexpression as expected is associated with resistance to apoptosis induced chemotherapy drugs whereas at times over expression may enhance apoptotic activity[80].The reason for such observation could be related to BAX as seen by various studies[80]. There was a correlation found between reduced BAX expression and resistance to chemotherapeautic drugs as opposed to increase BCL-2 levels. Thus it was BCL-2/BAX ratio which was more important in predicting tumor response[80]. Overexpression of BAX can negate the effect of BCL-2 and allow apoptosis to occur with chemotherapeutic drugs. Chang, et al.[81] found in their study that 54% BAX positive pretreatment biopsy had complete response and 29% BAX positive had partial response. Similarly Kudrimoti, et al.[82] concluded that BCL-2 positivity was seen amongst 60% of complete responders and 16% of partial responders.
Our study showed no correlation between BCL-2 expression and response but interestingly all 4 patients who had pCR had BCL-2 overexpression[80].

Thymidylate Synthase

Treatment with 5 FU (Fluorouracil) is now considered the standard of care for CRC. One of the main mechanism of action of this drug is competitive inhibition of thymidylate synthase (TS) which is a rate limiting enzyme in de novo synthesis of 2′-deoythymidine-5′-monophosphate which in turn is required for DNA synthesis[83]. When 5-FU is converted into its active metabolite it forms a stable complex with TS and its then that the activity of the enzyme is inhibited thus resulting in cell cycle arrest and death. Various genetic polymorphism have been described.

Various studies have evaluated the correlation between TS and the response to CRT in rectal cancer. Negri, et al. [84] showed that patients with high TS staining intensity had significantly better complete and partial response than those with low TS intensity. Whereas Bertolini, et al.[54] evaluated 91 patients receiving nCRT and found no correlation with TS staining. In contrast Saw, et al.[85] showed that post treatment biopsy negative for TS staining were associated with tumor down staging in nCRT group. A recently published meta analysis by Yang YC, et al.[86] aimed to clarify correlation between TS polymorphism and tumor response and they demonstrated that TS 2R/3R polymorphism was associated with better response to nCRT compared to both 1494del6 and nor 5' UTR expression. Study conducted at our institute aimed to find similar correlation of TS gene expression and polymorphisms with tumor response to nCRT with LARC. We found that patients with low expression group with a G>C single-nucleotide polymorphism (SNP) (i.e2R/3RC, 3RC/ 3RC) showed a significantly higher rate of tumor down staging, compared to patients in the high expression group without the SNP (i.e2R/3RG, 3RC/3RG, 3RG/3RG) [87].

K-RAS Oncogene

K-RAS mutation is one of the most common oncogene mutation implicated in CRC with approximately 30-50% of colorectal tumors known to have mutated(abnormal) KRAS thus implying that upto 50% of patients with CRC may respond to anti-EGFR antibody therapy[88]. Inspite of this, around 40-60% of patients with wild type KRAS tumors do not respond to treatment [88]. Most commonly implicated K-RAS mutation is located at codons 12 and 13 of KRAS gene on short arm of chromosome 12 (exon2). Many institutes routinely test K-RAS mutation prior to starting adjuvant treatment for colon cancer but its use as a biomarker after CRT is less known.

Studies by Clancy, et al.[89], Bengala C. et al.[90], and Gaedcke J. et al.[91] suggested no correlation with KRAS and tumor response after CRT. Whereas Duldulao. et al.[92] found that tumors with KRAS mutation had a lower incidence of pathological complete response than those with wild type KRAS. On contrary, Luna P. et al[93] suggested that those with wild type KRAS tumors had a better chance to be responsive than mutated tumors.
Interestingly, study by Martellucci J. et al.[88] demonstrated that having KRAS mutation does not confer any radio resistance and there is no significant correlation between pCR, tumor down staging, T downsizing or any cancer related mortality or overall survival and disease free survival in patients having KRAS mutation. They also suggested that patients with K-RAS codon 13 mutations may be relatively resistant to CRT and thus less likely to achieve pCR. Apparently, K-RAS gene appears in two forms; wild and non-wild forms, thus testing KRAS gene may be essential to decide on which type of chemotherapy target agent is suitable for which patient and tumor mutation.

Gene Expression

As reviewed above, single specific markers have limitations and to overcome these, DNA micro-array based gene expression-profiling technology is underway to analyze large number of genes at a time and to systematically search for a molecular biomarker to predict response to CRT. Various forms of genes has been analyzed in various studies. Watanabe, et al.[93] identified 33 genes with different expression amongst responders and non-responders post CRT with accuracy of 82.4%. Ghadimi, et al.[95] identified 54 genes that differed between responders and non-responders with accuracy of predicting tumor behavior in 83% patients.
Gantt, et al.[96] used 812 gene signatures and successfully identified non responders with sensitivity and specificity of 100% and using 183 gene signatures they identified non-responders with sensitivity and specificity of 33% and 100% respectively.

Similarly Kim, et al. [97] used 95 gene signatures and predicted response accuracy of 80%.
On the other hand, Brettingham-Moore et al[98] suggested that microarray tests are not robust enough for its use in rectal cancer. Microarray test do have several limitations. Firstly there is no uniformity of gene signatures, second there is no proper reproducibility and lastly the cost is a major limitation.

MicroRNA (miRNA)

miRNA are short non-coding single stranded RNAs usually around 18 and 25 nucleotides in length which induce post transcriptional gene silencing by mRNA degradation or translation blocking thereby regulating genetic expression and thus influencing many patho-physiological process [99,100]. A recently published review by Azizian A, et al.[100] mentions the role of miRNA in Rectal cancer. Slattery, et al. compared different tissue samples from both colon and rectum and they revealed molecular differences between colon and rectal cancer[101]. A study by Drebber, et. al.[102] suggested that CRT in LARC induces various alterations in miRNA expression in normal tissues and they are associated with positive response to treatment. They also described in 40 patients, relation between miR-145 expression levels and pathological tumor downstaging.

Svoboda *et al*[103] suggested that high levels of miR-124b and miR-137 were associated with poor response to CRT. Another study by same group demonstrated that 3 miRNAs miR-215, miR-190b and miR-29b-2 were overexpressed in nonresponders whereas let-7e, miR-196b, miR-450a, miR 450b-5p and miR-99a were down regulated in responders. Scarpati*et al[104]* identified 11 miRNAs(miR-1183, miR-483-5p,miR-622, miR-125a-3p, miR-1224-5p, miR-188-5p,miR-1471, miR-671-5p, miR-1909, miR-630, miR-765) which were significantly upregulated in patients with complete response and 2 (miR-1274b, miR-720) were down-regulated. Hotchi, et al.[105] on the other hand suggested a miR-223, a completely new miRNA expressed in patients tissue having a good response to CRT. Various other studies suggested various miRNAs in response to CRT but currently there are no well established miRNA biomarkers.

Current Miscellaneous Issues

The search for an ideal biomarker is still on and there are various ongoing studies and trials under evaluation. Various trials are considering Multidirectional Evaluation Model based on the principle that each marker has its own advantages and disadvantages and thus combining two or more may improve predictability of CRT response. Lambrecht, et al[106] are evaluating combination of PET-CT and DW-MRI. Maas, et al[107] evaluated clinical, radiological and endoscopic criteria and suggested wait and watch policy.

Jwa, et al[108] formulated a nomogram to predict ypN status post CRT in LARC using logistic regression analyses. Furthermore, Carpinetti P, et al[109] suggested use of liquid biopsies and personalized biomarkers in response to CRT using circulating tumor DNA(ctDNA) and suggested that they can be used as a tool for treatment. Another marker in study is Circulating cell-free (cf) nucleic acids in blood. Zitt, et al[110] studied amount of ctDNA in plasma in patients with CRT and observed a decreased level in responders and increased level in non-responders. Lastly, Teng F, et al[111] studied tumor-infiltrating lymphocytes, fork-head box P3,programmed death ligand-1, and cytotoxic Tlymphocyte–associated antigen-4 expressions before and after CRT and demonstrated that pre CRT CD81TILs, CD41TILs, and MDSC-TILs are sensitive predictive markers for response to CRT, and high CD81TILs are associated with good prognosis.

Conclusion

Biological biomarkers are in the evolving stage with new upcoming trials to promote their utility in the field of rectal cancer particularly in evaluating tumor response after CRT. At present CEA marker and p53 as well as VEGF along with imaging studies act as the main bulk of debates since their contribution are relevant in many aspect of tumor nature in terms of tumor response, overall prognosis and it may help to clear management agenda for each individual patient. Therefore, well-organized randomized trial to investigate and compare these biomarkers is in the near future.

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Conflict of Interest

All authors declare no conflict of interest.

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Table (1): Results of Tumors Response in Relation to Molecular Biomarkers

Author	Year	No.	Patients	Marker	Outcome
Kim [70]	2006	183	cT3-T4 or N+)	EGFR	Down staged 53% Increased tumor down-staging observed in a low level of EGFR expression
Jang [17]	2011	109	Advaned rectal cancer	CEA	3-year DFS rate was significantly better if preCEA<3.5 (94.7 vs. 52.6%)(P < .001)
Peng [113]	2012	116	Stage III rectal cancer	VEGF	VEGF was identified among 74.1% and has shown: 1- Increased incidence of distant metastasis (19.8 vs. 3.3 %, p00.039), 2-Decreased 3-year DFS rate (96.7 % vs. 72.7 %, p<0.01)
Sprenger [113]	2013	126	Stage II/III rectal cancer	CD133	High expressing (CD133+) after pre CRT had higher residual tumor Lower tumor regression, reduced DFS (P < .01).
Jang	2013	350	CRC	VEGF genotypes	VEGF -2578CA genotype had poor prognosis in RC

[68]					Combined VEGF -2578CA+AA/-1154GG genotype has poor OS
Yoon	2014	50	Stage II or III	TIMP-1,UPAR,VEGF	Post CRT TIMP-1 level ≤ 204.5 ng/mL was associated with pCR
[114]				EGFR	and with MRG group ($P < 0.01$).

CEA: carcino-emboryonic antigen, CD: …… CRC: colorectal cancer, CRT; chemo-radiotherapy, DFS: disease free survival, EGFR: epidermal growth factor receptor, VEGF: vascular endothelial growth factor, OS: overall survival, TIMP: tissue inhibitor of metalloproteinases, UPAR: urokinase plasminogen activator, MRG: Mandard regression grade.