Advances in Modern Oncology Research

doi: 10.18282/amor.v1.i1.17

ORIGINAL RESEARCH ARTICLE

Osteopontin level correlates negatively with tumor shrinkage in neoadjuvant chemoradiation of locally advanced rectal cancer

Mirna Primasari[1*], Soehartati A. Gondhowiardjo[1], DiahRini Handjari[2], Sahat Matondang[3], Adang Bachtiar Kantaatmadja[4]

[1]Radiotherapy Department, Dr. Cipto Mangunkusumo General Hospital, Jakarta, Indonesia
[2]Anatomical Pathology Department, Dr. Cipto Mangunkusumo General Hospital, Jakarta, Indonesia
[3]Radiology Department, Dr. Cipto Mangunkusumo General Hospital, Jakarta, Indonesia
[4]Department of Public Health, University of Indonesia, Jakarta, Indonesia

Abstract: **Background and Purpose**: Neoadjuvant chemoradiation (CRT) is the mainstay treatment for locally advanced rectal carcinoma. However, the response is varied due to many factors, including tissue hypoxia. Osteopontin (OPN) is an emerging endogen hypoxic marker with significant correlation towards tumor pO2, also a more accurate chronic hypoxic marker compared to carbonic anhydrase IX (CAIX), glucose transporter 1 (GLUT1), and lactate dehydrogenase A (LDHA); but as far as we know there is no research that measured OPN quantity in rectal cancer tissue and correlated it with tumor shrinkage response in neoadjuvant CRT. **Materials and Method**: Fourteen patients that met the inclusion and exclusion criteria were analyzed retrospectively. Imaging was evaluated for tumor shrinkage percentage and categorized based on Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 criteria. OPN level quantitative results from rectal cancer tissue were obtained using ELISA method. **Results**: The mean OPN concentration was 0.568 ± 0.26 ng/mL. There was a significant strong negative correlation ($r = -0.630$, $p = 0.016$) between the OPN level and tumor shrinkage. OPN cut off value of ≥0.538 ng/mL predicted non-responsiveness of tumor shrinkage in neoadjuvant CRT with 100% sensitivity and 81.8% specificity. **Conclusion**: Hypoxia occurred in locally advanced rectal carcinoma patients. Higher level of OPN correlates negatively with tumor shrinkage, proved worse response of CRT given.

Keywords: rectal cancer; osteopontin; hypoxia; neoadjuvant chemoradiation

Citation: Primasari M, Gondhowiardjo SA, Handjari DR, et al. Osteopontin level correlates negatively with tumor shrinkage in neoadjuvant chemoradiation of locally advanced rectal cancer. Adv Mod Oncol Res 2015; 1(1): 56–61; http://dx.doi.org/10.18282/amor.v1.i1.17.

*Correspondence to: Mirna Primasari, Radiotherapy Department, Dr. Cipto Mangunkusumo General Hospital, Jakarta, Indonesia. mirnaprimasari.md@gmail.com.

Received: 31st July 2015; **Accepted**: 31st August 2015; **Published Online**: 6th October 2015

Multimodality treatment is an evidence based therapy of locally advanced rectal carcinoma, including surgery, radiation therapy, chemoradiation (CRT) and chemotherapy. Treatment of choice and treatment sequencing can be different for each condition of rectal cancers from clinician judgement/perspectives. Neoadjuvant CRT combines adjusted low dose chemotherapy as radiosensitizer and radiation therapy which is done before surgery. It is proven to be more effective compared to adjuvant CRT which is done after surgery, in locally advanced rectal cancer. It provides better result in local control and minimizing treatment toxicity. It is also associated with tumor downstaging, increased tumor resectability, significantly higher rate of pathologic complete response, less advanced primary tumor (pT) and regional lymph nodes (pN) stage, and fewer cases with venous, perineural or lymphatic invasion[1]. According to the latest National

Comprehensive Cancer Network (NCCN) guidelines version 3.2015, neoadjuvant CRT has been accepted as the standard therapy for locally advanced rectal cancer with evidence level, Category 1. Category 1 is high-level evidence where there is uniform NCCN consensus that the intervention is appropriate[2].

However, the CRT response is varied between different locally advanced rectal cases due to many factors. Hypoxia decreases chemotherapy and radiation sensitivity through direct and indirect mechanisms. For decades, hypoxia has been studied and proven to be bad prognostic factor in cancer therapy[3,4]. Identifying the predictive biomarkers of response before therapy could be useful to indicate which patients will benefit from "hypoxia targeted therapy"[5].

Osteopontin (OPN) is an emerging endogen hypoxic marker. Its level is increased in several carcinomas, such as lung, breast, gastrointestinal, ovarium, prostate, pancreas, colon and rectal cancers. In cervical carcinomas, the OPN level elevates 90 times compared to normal tissue. In breast cancer, high level of OPN correlates with bad prognosis[6]. It also correlates with prostate cancer recurrence in 72 months[7].

Through this study we aim to prove that OPN which characterizes hypoxia, is a bad prognostic towards tumor shrinkage in neoadjuvant CRT. It presents itself in locally advanced rectal cancer, therefore quantitative measurement of OPN could be used as one of the examinations to identify which patients would need and benefit from "hypoxia targeted therapy" in order to increase the clinical outcome.

Materials and methods

All rectal cancer patients who were referred to the Radiotherapy Department, Dr. Cipto Mangunkusumo General Hospital were screened from medical records. Inclusion criteria were all locally advanced rectal carcinoma patients who received neoadjuvant CRT in the Radiotherapy Department with consisted of long course radiotherapy (total dose 46–50 Gy in 23–25 fractions or biologically equivalent dose) and low adjusted dose of chemotherapy as radiosensitizer. All the patients went through radiology imaging and also pathology review, both performed in Dr. Cipto Mangunkusumo General Hospital to ensure that the evaluation could be done. Patients would have been excluded if the data or tissue samples had not been sufficient or if there were comorbidities affecting the patients' vascular condition (diabetes mellitus, cardiovascular disease, hematologic disorders, hypertension, chronic kidney disease). Also the time interval for imaging post-CRT should be at least four weeks. There were fourteen patients screened from January 2012 until January 2015 who met all the inclusion and exclusion criteria above. Patients' characteristics that have met the inclusion and exclusion criteria are described in *Table 1*.

Table 1 Patients' characteristics

Characteristics	Data
N	14
Age (y)	
Mean ± SD	46.86 ± 3.45
Median (min–max)	43.5 (26–70)
Age by group	
26–45	8 (57.1%)
45–65	4 (28.6%)
>65	2 (14.3%)
Gender	
Male	6 (42.9%)
Female	8 (57.1%)
BMI	
<18.5	7 (50%)
18.5–23	4 (28.6%)
23–25	0
>25	3 (21.4%)
KPS	
>70	11 (78.6%)
≤70	3 (21.4%)
Stage	
II	2 (14.3%)
III	12 (85.7%)

Abbreviations: BMI = body mass index. KPS = Karnofsky performance status. From the 14 samples acquired, mean age was 46–47 years old with the median age is 43.5 years old and from the age classification more patients were in young age rather than old age. 50% patients were underweight and 85.7% were in stage III. All patients underwent CRT with capecitabine oral with daily intake scheme.

The independent variable was the OPN level while the dependent variables were tumor shrinkage percentage and the category of CRT response.

A computed tomography (CT) or magnetic resonance imaging (MRI) should be performed before and after CRT. The CT or MRI specification should be, and was the same, pre and post therapy to ensure unbiased evaluation, reviewed by one radiologist specializing in abdomen and pelvis imaging. Evaluation for tumor shrinkage percentage was conducted using tumor unidimensional measurement according to Response Evaluation Criteria in Solid Tumor (RECIST) 1.1 protocol. Nodal evaluation (N) or distant metastasis (M) were not done. The tumor shrinkage percentage was then categorized using RECIST 1.1 criteria to evaluate CRT response.

Rectum tissue was obtained from formalin-fixed, paraffin-embedded (FFPE) tissue. Before the enzyme-

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linked immunosorbent assay (ELISA) procedure, a pathology review was done by a pathologist (colorectal specialist), to rate the percentage ratio of the tumor in normal/overall tissue since rectal cancer tissue is usually very heterogeneous. The percentage of tumor is used as correction factor for calculating OPN level in rectal cancer tissue.

Deparaffinization procedure

Deparaffinization was performed a day before the ELISA procedure. The FFPE tissue was cut 25 µm thick and put into microcentrifuge tubes. The paraffin slices were washed with 1 mL of xylene, mixed in a vortex mixer for 10 s and incubated in room temperature for 45 min. After that, the solution was centrifuged at 1000×*g* for 1 min to allow sedimentation. The supernatant was then discarded. The entire process was repeated two more times for an incubation period of 30 min. Using the same procedure, 1 mL of ethanol was added to wash the xylene with 100%, 70% and 40% concentration subsequently for 30 min each. The steps were repeated twice using aquadest for 15 min each.

Protein extraction

1:1 phosphate buffered saline (PBS) solution was added. Using the glass on glass homogenizer, the tissue was minced ±30 strokes until there were no tissue chunks seen. To break the cell membranes further, the solutions were frozen and thawed twice. They were centrifuged at 5000×*g* for 5 min in 4°C and the supernatant was removed carefully to be used as sample for ELISA procedure.

ELISA procedure

The ELISA kit used was from Cloud-Clone Corp assembled by USCN Life Science Inc. SEA899Hu. Standard solutions were prepared using dilution according to the manual and mixed well using a shaker. 100 µL of the supernatant were added from 14 samples along with the standard solutions onto the plate that had been coated with the antibody. After being incubated in a 37°C environment for 2 h, reagent A was added before another incubation, for 1 h. The plate was washed first before reagent B was added and incubated for 30 min before the final wash. 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate was added right before the plate was covered with plate sealer to avoid the light for 15–25 min. Stop solution had to be added precisely after 25 min and the OPN levels were immediately read with a spectrophotometer using a 450 nm wavelength. There was no modification applied to the protocol from the manufacturer.

Ethics statement

This study was approved by the Ethics Committee, Medical Faculty, University of Indonesia.

Results

Radiology and laboratory evaluation

Radiology evaluation showed a wide range of tumor shrinkage percentage after CRT, from −81.2% to 52.4%, with a mean value of tumor shrinkage percentage of 7.89% (*Table 2*).

Table 2 Radiology results

Result	Data
Tumor size (cm)	
Mean ± SD	
Before CRT	4.42 ± 1.33
After CRT	4.08 ± 3.06
Median	
(min, max)	
Before CRT	4.05 (2.9–7.0)
After CRT	3.6 (1.7–8.7)
Tumor Shrinkage (%)	
Mean ± SD	
Median	7.89 ± 35.79
(min, max)	12.8 (−81.20–52.40)

The mean value of OPN presented in all rectal cancer tissue samples is 0.568 ± 0.26 ng/mL with the lowest level being 0.329 ng/mL and 1.248 ng/mL being the highest, as shown in *Table 3*. No sample had OPN level which was low enough to be undetected from the assay.

Table 3 Laboratory results

Result	Data (ng/mL)
Mean ± SD	0.568 ± 0.26
Median (min, max)	0.49 (0.329–1.248)

Correlation of OPN levels with tumor shrinkage

Numeric variables of OPN levels were analyzed statistically using Statistical Product and Service Solutions (SPSS) Software version, 21, Pearson correlation with bootstrap technique (*Table 4*). There was a statistically strong significant correlation between OPN level and tumor shrinkage percentage ($r = -0.630$, $p = 0.016$) as shown in *Table 4* and *Figure 1* that shows scattered plots of correlation between OPN levels and tumor percentage.

doi: 10.18282/amor.v1.i1.17

Table 4 Correlation between OPN levels and tumor shrinkage percentage

Pearson Correlation	
r	−0.630
p	0.016
Reference	
Correlation (*r*)	**Interpretation**
0.0 – <0.2	Very weak
0.2 – <0.4	Weak
0.4 – <0.6	Intermediate
0.6 – <0.8	Strong
0.8–1	Very strong
***p* value**	
p <0.05	Significant correlation between two tested variables
p >0.05	No significant correlation between two tested variables
Correlation direction	
+ (positive)	Linearly correlated, bigger value from one variable increased value of the other variable
− (negative)	Inversely correlated, bigger value from one variable decreased value of the other variable

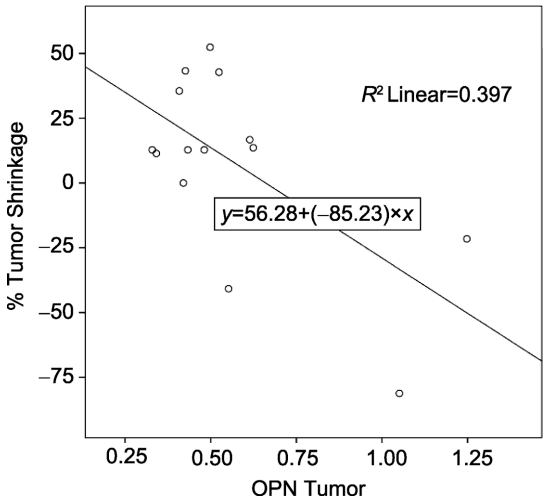

Figure 1 Scattered plot of correlation between OPN levels (*x* axis) and tumor shrinkage percentage (*y* axis)

Radiologic response using RECIST 1.1 criteria

Tumor shrinkage percentage was categorized according to RECIST 1.1 criteria:

Complete response (CR) :	no residual lesion.
Partial response (PR) :	>30% regression.
Progressive disease (PD):	>20% tumor enlargement than baseline or new lesion.
Stable disease (SD):	meet no criteria above.

Radiology evaluation was performed and found that four patients (29%) had partial response, seven patients (50%) had stable disease and 3 patients (21%) had progressive disease. Five out of fourteen patients (35%) managed to undergo a resection procedure after CRT while 65% of the patients could not.

Cut-off value of OPN level

Subanalysis was done for CRT response. The partial response and stable disease were categorized as responsive towards CRT while the progressive disease group was categorized as non-responsive. Eleven patients (78.57%) were in the responsive category, while three patients (21.4%) were in the non-responsive category (*Table 5*). The data was then analyzed against OPN levels using SPSS for Receiver Operating Curve (*Figure 2*).

Table 5 CRT response

CRT Response	*N* (%)
Responsive	11 (78.57%)
Non-responsive	3 (21.4%)

According to the RECIST 1.1, the therapy response was categorized into 3 criteria: partial response, stable disease and progressive response. Authors categorized the progressive response as non-responsive and the rest as responsive towards CRT.

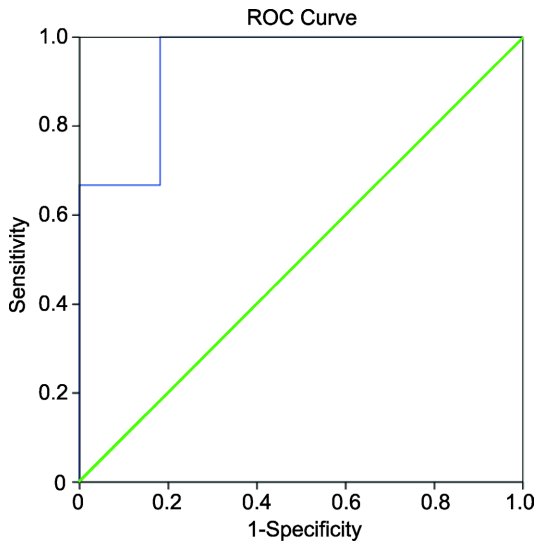

Figure 2 ROC for non-responsive category against CRT. Area under the curve (AUC) from the analysis was 0.939 with $p = 0.024$ which indicated a very strong interpretation between the variables. The cut off for OPN value was ≥0.538 ng/mL with 100% sensitivity and 81.8% specificity

Discussion

In this preliminary study, the result showed that all locally advanced rectum cancer tissue expressed OPN pro-

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tein that indicated a hypoxic state of rectal tumor tissue. In a separate previous study, OPN expression was detected using immunohistochemistry (IHC) in 45.83% of the studied colorectal (CRC) cases, while normal colonic epithelium was immunonegative. In normal CRC gland epithelia, OPN only displayed weak staining in < 10% of the cells therefore all the cases were determined as negative[8].

These results are consistent with other studies showing up-regulation of OPN in certain neoplastic epithelia, while normal epithelia exhibited low levels or even negative expression. In the study by Li et al., 49.4% OPN expression of CRC cases was observed while normal colorectal gland epithelia revealed negative expression[9]. Moreover, Rhode et al. found slightly higher rates of OPN expression (56%) in CRC cases[10].

There was no previous research which involves the OPN protein quantitative value from rectal cancer tissue or rectal normal tissue. We have not been able to calculate the increase of OPN values in rectal carcinoma since there was no data for normal rectal tissue OPN quantitative values. Further research will be required.

The cut off value of 0.538 ng/mL could predict the non-responsiveness of tumor shrinkage in neoadjuvant CRT. Three out of three patients (100%) who were non-responsive towards CRT showed OPN value ≥0.538 ng/mL while only one out of 11 patients (0.09%) who was responsive towards CRT showed OPN value ≥0.538 ng/mL (0.624 ng/mL).

OPN is a phosphorylated glycoprotein found in all body fluids, extracellular matrix components, proteinaceous matrices of mineralized tissues and overexpressed in tumors[11]. OPN itself is a part of small integrin-binding ligand N-linked glycoprotein (SIBLING) whose gene location has been mapped to the long arm of chromosome 4, close to other SIBLING: bone sialoprotein (BSP), dentin matrix protein I (DMPI) and dentin sialophosphoprotein (DSPP) genes[12].

The molecular pathway of OPN expression and activation has not been completely understood. A number of theories from previous studies on several types of cancer conclude that hypoxia-inducible factor (HIF) 1α increases the transcription of hypoxic markers GLUT1, CA9, VEGF, etc., including OPN, as shown in *Figure 3*[13]. HIF1α activates OPN through the prostaglandin (PG) pathway as stated in the study on gastric cancer (*Figure 4*). Besides this, from *Figure 5*, we can understand that transcription of OPN has several downstream pathways, through wild-type Ras that will subsequently activate protein kinase B to Ras enhancer to in turn, increase

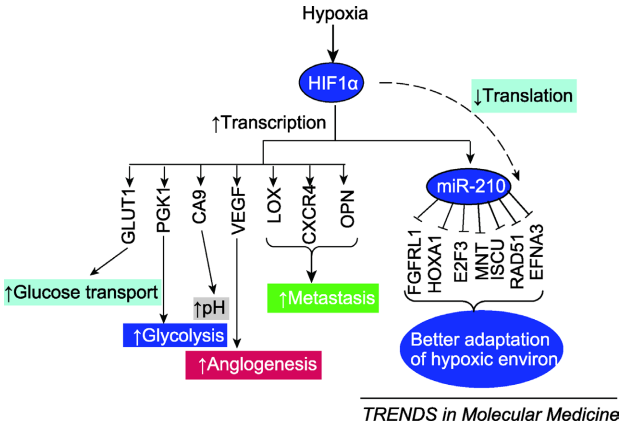

Figure 3 OPN transcription from HIF1α[13]

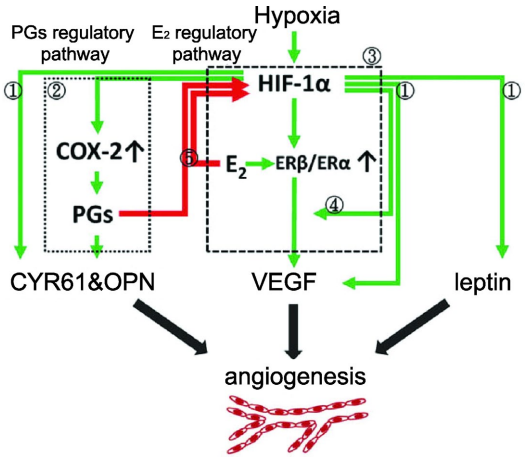

Figure 4 Osteopontin pathway from HIF1α through prostaglandin pathway in gastric cancer [Source: Frontiers in Bioscience, 2015, available from https://www.bioscience.org/2015/v7e/af/ 736/fig3.jpg]

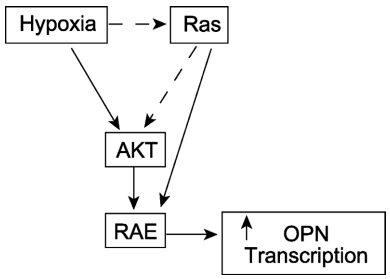

Figure 5 Downstream pathway through wild-type Ras[14]. OPN binds to integrin and non-integrin receptors in epithelial and/or endothelial cells to activate kinases and transcription factor that leads to angiogenesis, proliferation/tumor growth, invasion and metastasis. OPN also proves to be synergistic and has positive correlation with VEGF in angiogenesis[16].

OPN transcription[14]. A study specially done in colorectal cancer also proved that OPN was a transcriptional target of the wingless-type MMTE (WNT) pathway[15].

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Hypoxia contributes in radio- and some chemo-resistance through direct and indirect mechanisms[1]. A study by Overgaard et al. provides evidence of a predictive value of OPN protein, which has been shown to be associated with tumor hypoxia[5].

OPN correlates with tumor tissue partial oxygen pressure (pO2) compared to CA9, BNIP3L, connective tissue growth factor, ephrin A1, hypoxia inducible gene-2, dihydrofolate reductase, galectin-1, IkB kinase β, and lysyl oxidase in head and neck experimental in 2007[6]. Hypoxic condition increases stimulated secretion of OPN *in vitro* and it was proven from previous research that the plasma OPN level is inversely correlated with pO2. OPN level will get higher along with worsening hypoxic condition. It was concluded that high OPN concentrations predicted clinically relevant, modifiable hypoxia-induced resistance to radiotherapy and this finding could help to identify patients who will benefit from treatment with a hypoxia modifier[5].

Conclusion

From this pilot study, we found that OPN presents in locally advanced rectal cancer tumor tissues. OPN, as an endogen hypoxic marker, suggested hypoxic condition that will inversely correlate with tumor shrinkage response in neoadjuvant CRT. Cut off value of OPN in rectal tissue could help to predict tumor shrinkage response but further study and analysis with larger sample is required. It would also be very interesting to do a prospective study for the next research to get more data for analysis (for example: correlation of serum OPN with tumor OPN and tumor percentage shrinkage, OPN levels before and after therapy, etc.).

Conflict of interest

The authors declared no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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