

RESEARCH ARTICLE

Evaluation of *Methylobacterium radiotolerance* and *Sphingomonas yanoikoaie* in Sentinel Lymph Nodes of Breast Cancer Cases**Hamid Reza Yazdi¹, Abolfazl Movafagh^{2*}, Fateme Fallah³, Shohreh Alizadeh Shargh², Neda Mansouri², Atefeh Heidary Pour⁴, Mehrdad Hashemi⁵****Abstract**

It has been established that different kinds of bacteria agents are involved in various cancers. Although the mechanism of tumorigenesis is not clearly understood, there is evidence for the presence of bacteria within tumors, with at least a progression effect for some bacteria that prepare suitable microenvironments for tumor cell growth. The aim of current study was to evaluate bacterial dysbiosis in sentinel lymph nodes of breast cancer patients. One hundred and twenty three fresh-frozen sentinel lymph nodes and a corresponding number of normal adjacent breast tissue specimens and five normal mastectomy samples were investigated employing RT-PCR. In addition using genus-specific primers were applied. There was a significant differences as presence of *Methylobacterium radiotolerance* DNA recorded between patients and normal control group ($p= 0.0$). Based on our research work, further studies into the role of microbes in breast cancer would be of great interest.

Keywords: Breast cancer - sentinel lymph nodes - *Methylobacterium radiotolerance* - *Sphingomonas yanoikoaie*

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Introduction

The association between the development of cancer and bacterial agents, has been proved in recent years (Mager, 2006). Some evidence shows that relation between helicobacter pylori infection and gastric cancer and some other type of malignancies such as lymphoma were reported (Cover and Blaser, 2009; Polk and Peek, 2010). Other types of bacteria has been shown that present their infection in tumor tissues (Toso et al., 2002; Heppner et al., 1978). Indeed, some bacteria replicate and establish specifically within tumor cells (Baban et al., 2010).

Infection with microbial agents is linked to higher risk of malignancy with significant attribution of bacterial infections (Pujol and Devesa, 2005; Ord and Blanchaert, 2001). Some species of bacteria, like salmonella typhi is in association with gall bladder cancer (Vaishnavi et al., 2005), streptococcus bovis and colon cancer (Littman et al., 2004), and chlamydia pneumonia reported with lung cancer (Kuper et al., 2000).

Bacteria can change the immune system status and suppress its effective response and even evade to immune system (Kuper et al., 2000). By long-term infection with

bacteria as chronic disease, also bacteria can produce some dangerous agents such as toxins that able to change cell cycle regulation and as the consequence, alter cell growth (Koyi et al., 2001).

The anaerobic bacteria (*Peptostreptococcus*) detected in the lymph nodes have been detected in gastrointestinal tumor tissue, suggesting that the oral mucosal defect can induce translocation of anaerobes into the neighbor lymphatic nodes (Sakamoto et al., 1999). In another study, some of microbial biomarkers that present in the salivary, are specific for pancreatic cancer (Sjostedt et al., 1988).

In recent study, Xuan and his co-workers, investigated *Methylobacterium radiotolerance* and *Sphingomonas yanoikuyae* in the breast cancer tissue with adjacent normal tissue. They found relative abundances of these two bacterial species were inversely correlated in paired normal breast tissue but not in tumor tissue, showing the dysbiosis is associated with breast cancer (Caiyun et al., 2014).

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Material and Methods

Ethics statement for obtaining breast tissue specimens

One hundred twenty three fresh-frozen sentinel lymph node of breast cancer, and 123 normal adjacent breast tissue and 5 normal mastectomy samples were obtained in accordance with institutional requirements according to health ministry guidelines. The study protocol was approved by the Ethical Committee of the Pishva Islamic Azad University. Written consent was specifically waived by the approving of pathological consents. Extracted bacteria DNA from Methylobacterium Radiotolerance and Sphingomonas Yanoikuae was obtained from microbiology department of Radboud University, Nijmegen, Netherland.

Genomic DNA (gDNA) extraction from fresh-frozen tissue

Total genomic DNA was extracted from FFPE breast tissues using Sigma, GenElute™ Mammalian Genomic DNA Miniprep Kit Protocol (G1N10) per manufacturer's instructions with slight modifications. Purified gDNA was eluted twice from the column using ultrapure water. All extractions were performed in a designated clean (pre-PCR) room. RT-PCR, Sybr green for bacterial copy numbers RT-PCR was performed using the genus-specific primers 5F (forward, 5'- AACGTGCCTTCTGGTCTGG -3') and 8R (reverse, 5'- TCCGGCAGTAAACCTTCCC-3') were used to quantify *M. radiotolerans* (AY616142.1 (71.153), amplicon size 83bp) and, Sph-spt 694F (forward, 5'- AAGGCGACGATCCTTAGCTG-3') (AF331661.1(2264493... 2264177, 317 bp) and Sph-spt 983R (reverse, 5'- CGGCTTTCACCTCTGACTT-3') were used to quantify *Sphingomonas* microbial

DNA extracted from fresh frozen tissue. Primers designed were using "Primer blast 3, NCBI" software. The reaction condition was as follows: Sybr buffer 10 µl, Primer F (10pmol/ µl) 0.5 (10pmol/ µl), Primer R (10pmol/ µl) 0.5 Primer R(10pmol/ µl), ddH₂O µl, DNA 2 µl, final volume 20 µl. The cycling program for *Methylobacterium Radiotolerance* was Pre Denaturation 95°C, 6 min, one Denaturation 95°C, 50 sec, 2 Annealing/ Extension 56°C, 59 sec, Melting Curve analysis 70-95, 0.5S/degree and for *Sphingomonas Yanoikuae* was; Pre Denaturation 95°C, 5 min, 1 Denaturation 95°C, 60 sec, 2 Annealing/ Extension 54, 60 sec, Melting Curve analysis 70-95, 0.5S/degree (Figure 1).

All samples from pyrosequencing were also assessed for bacterial sequence confirmation (Subjects 1-5, the positive RT-PCR products for *Methylobacterium Radiotolerance*, and 4 positive samples for *Sphingomonas Yanoikuae*) (Figure 2). DNA from healthy specimens was obtained from patients undergoing reduction mammoplasty, with no evidence of breast cancer. Bacterial copy numbers were normalised by the total amount (µg) of extracted DNA of beta-actin house keeping gene. Samples were randomized and processed in a blinded manner.

Statistical analysis

All data were analyzed with Statistical Product and Service Solutions (SPSS) software (version 17.0, SPSS Inc., Chicago, IL, USA). Participants were categorized into three groups: Breast cancer, normal adjacent breast tissue and normal mastectomy samples were obtained.

Comparisons in stages among three groups were performed using the Chi square test and comparisons between each two groups were performed using the partition of Chi square test. Differences were considered

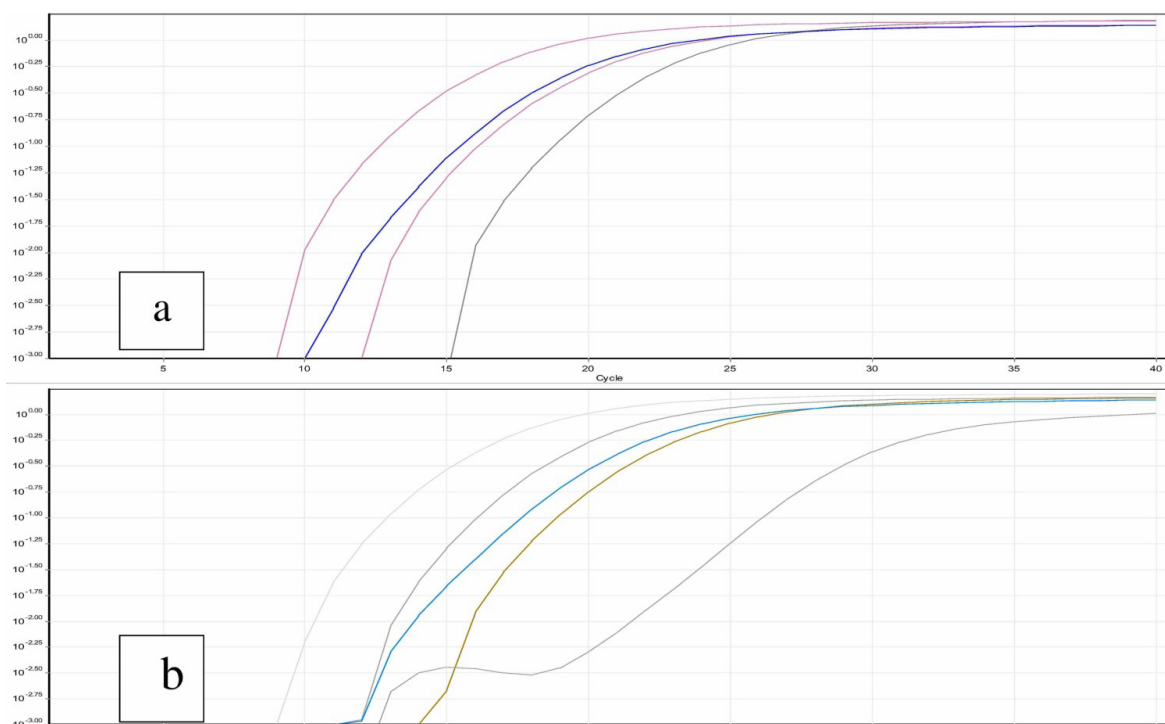


Figure 1. The Sybr Green RT-PCR Graphs for *Methylobacterium radiotolerans* and *Sphingomonas Yanoikuae*, DNA Sample, Figure 1a: *Methylobacterium Radiotolerance* Figure 1b: *Sphingomonas Yanoikuae*.

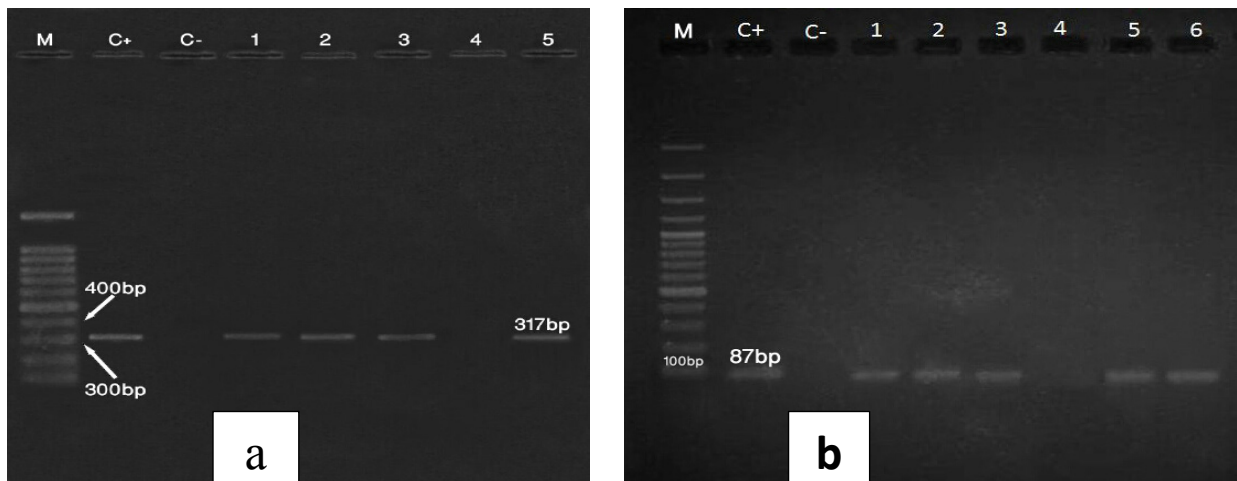


Figure 3. Electrophoresis of RT-PCR Products in 2% Agarose Gel, Ladder for Sphingomonas yanoikoea is 100bp (a) and for Methylobacterium radiotolerance is 50bp (b),c1: Positive Bacterial DNA Control, c2: Normal Mastectomy Breast Tissue,no 2,2,3 Positive Samples from Sentinel Lymph Nodes, No4: Normal Breast Tissue, No 5,6: Positive Sentinel Lymph Node.

as statistically significant if the P value was <0.05.

Result

In the present study, 123 sentinel lymph node that were pathologically negative, and 123 normal adjacent breast tissue has evaluated. DNA from Methylobacterium Radiotolerance and SphingomonasYanoikoea as positive control was analyzed. From 123 sample (one of them is excluded because of low quality), 5 sentinel lymph nodes has 5 sample showed Methylo bacterium Radiotolerance DNA and from 123 normal adjacent breast tissue, 4 sample showed SphingomonasYanoikoea DNA (Figure 3).

Evaluation of RT-PCR results with specific primers for Methylo bacterium Radiotolerance

In statistical analysis between cancer and normal samples, there was a significant difference as presence of Methylo bacterium Radiotolerance DNA in tumor samples (p= 0.0).

In this survey, 3% (100 sample) of cancer samples were in stage 1, had not the bacterial content and non of positive samples were in stage 1. Also of positive 2.1% (2 samples) and 97.9% 993 negative samples were in stage 2. From 12.5 % (3 sample) of positive samples and 95.5 % (117 sample) of negative samples were stage 3. In Pearson chi-square test, there was a significant relation between stage and DNA of Methylobacterium Radiotolerance,

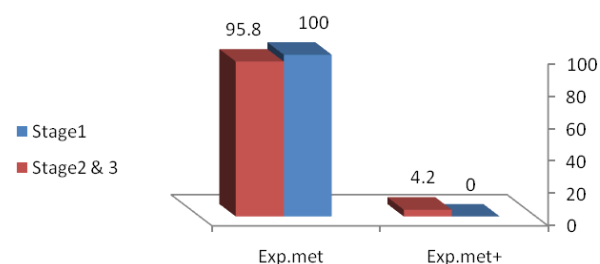


Figure 4. Relation between DNA of Methylobacterium Radiotolerance in Sentinel Lymph Node Samples of Breast Cancer and Stage of Tumor

means that in higher stages, the positive results were significantly higher that lower stages of breast cancer (p<0.01) (Figure 4).

The correlation between DNA of methylobacteriumradiotolerance with breast tumor grade

In 6.4% (3 samples) of positive samples and 93.6% (44 sample) of negative samples, were in grade 1 and from 2.7% (2 sample) positive samples and 97.3% (73 sample)

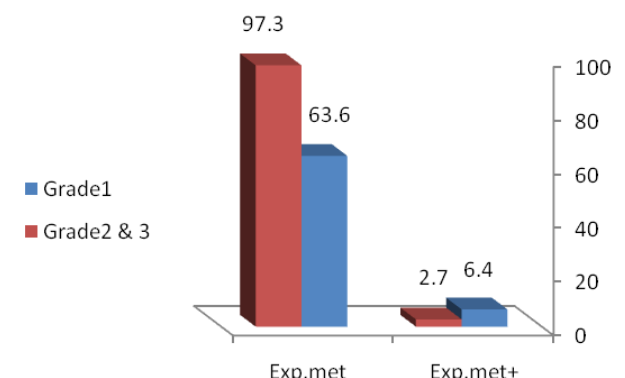


Figure 5. Relation between DNA of Methylobacterium Radiotolerance in Sentinel Lymph Node Samples of Breast Cancer and Grade of Tumor

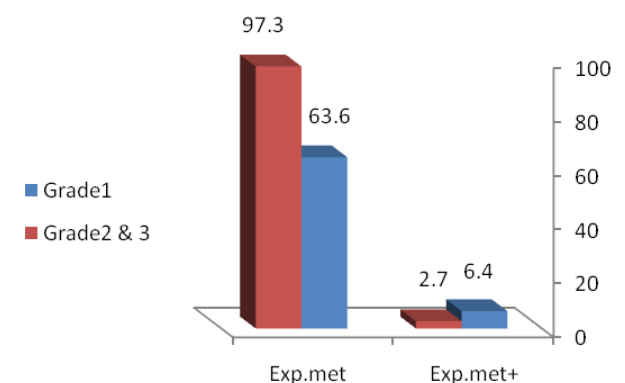


Figure 6. Relation between DNA of Sphingomonas Yanoikoea in Sentinel Lymph Node Samples of Breast Cancer and Stage of Tumor

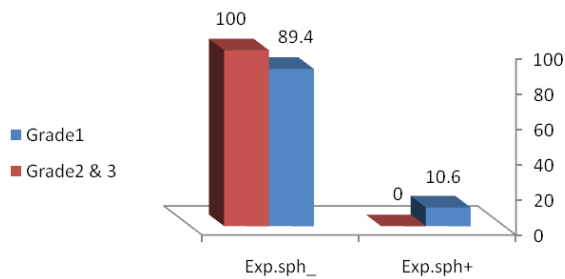


Figure 7: Relation between DNA of *Sphingomonas yanoikoa* in Sentinel Lymph Node Samples of Breast Cancer and Grade of Tumor.

negative samples were in grade 2. In Pearson chi-square test, there was not significant difference between presence of *Methylobacterium* Radiotolerance DNA and grade of tumor ($p=0.37$) (Figure 5).

Evaluation of RT-PCR results with specific primers for *Sphingomonas Yanoikoa*

In statistical analysis between cancer and normal samples, there was a significant difference as presence of *Methylobacterium* Radiotolerance DNA in tumor samples ($p=0.0$).

The correlation between DNA of Sphingomonas Yanoikoa with breast tumor stage.

In this study, 33.3% (1 sample) of positive cancer samples and 66.7% (2 sample) with negative result, were in stage 1. Also of positive 4.2% (4 samples) and 95.8% (114 sample) negative samples were in stage 2 and 3. In Pearson chi-square test, there was not a significant relation between stage and DNA of *Sphingomonas Yanoikoa* ($p=1.000$) (Figure 6).

The correlation between DNA of sphingomonas yanoikoa with breast tumor grade

In this study, 6.4% (3 sample) of positive cancer samples and 93.6% (44 sample) with negative result, were in grade 1. Also of positive 2.7% (2 samples) and 97.3% (73 sample) negative samples were in grade 2 and 3. In Pearson chi-square test, there was not a significant relation between stage and DNA of *Sphingomonas Yanoikoa* ($p=0.372$) (Figure 7).

In assessment for locality of patients (urban-rural) or metastasis occurrence after 3 months of sampling, there were no evidence for relationship between *Methylobacterium* Radiotolerance and *Sphingomonas Yanoikoa* content with them ($p=1.0$) (data not shown).

Discussion

The bacterial involvement in various kinds of cancer is established in several studies (Mager, 2006). Screening researches for presence of bacteria in cancer tissues in histologically different kinds of tissue, can reveal new aspects for this relation. Some bacteria can adapt with tumor microenvironment, serves the area for finding the exact mechanism and bacterial potential in tumorigenesis.

In the current study, the results revealed that the significant difference for presence of *sphingomonas yanoikoa* in normal tissue and also, presence of methyl bacterium radiotolerance in cancer tissue ($p=0.0$).

Moreover, in this study, the correlation between tumor's pathologic markers such stage, grade, current metastasis and locality of patients for living region all positive samples is evaluated, and it revealed that methyl bacterium radiotolerance in breast cancer is more prevalent in higher stages ($p<0.01$) but not in correlation with tumor's grade ($p=0.37$). This insignificant value maybe due to some sentinel lymph nodes had no grade as pathological report and discarded further from analytical tests. Also, in evaluation of presence of *sphingomonas yanoikoa*, with tumor's characteristics, there were not the significant correlation between the presence of this bacteria with tumor's stage and grade that the reason of this, maybe due to absence of this bacteria in tumor samples ($p=0.1$ $p=0.37$ respectively).

The results of statistical analysis for presence of *methylobacterium* and *sphingomonas* with locality and current metastasis, was not significant ($p=1.0$) that the reason of such finding maybe the result of limited sample number that is usual for sentinel lymph node sample.

In previous studies, it has been shown that the strongest relation between bacterial infection and tumorigenesis, is for *helicobacter pylori* (Samaras et al., 2010). The second importance is for *salmonella typhi*, causative agent of typhoid and can transport the bacteria chronically to bile ducts.

Recently in epidemiologic studies, it has been proved that the carriers of *Salmonella Typhi*, have the risk for gall bladder carcinoma 8.47 time and 6 times for hepato-biliary carcinoma greater than patients that had acute disease and cleared completely from bacteria (Wroblewski et al., 2010).

Moreover, recent studies has revealed that diseases like cancer need to genetical susceptibility that along with primary and secondary progression factors, can promote environmental malignancy (Heidari et al., 2014).

According to several studies, long term infection with *Chlamydomphila pneumonia*, can increase the risk for lung cancer (Caygill et al., 1994).

The mechanism underlying the changes in tumor cells for bacteria that cause the localization of bacteria in tumor tissues recorded in the literature (Lazcano-Ponce et al., 2001).

Also in evaluation of oral squamous cell carcinoma (OSCC), the DNA content of saliva in healthy persons and patients for *capnocytophagagingin valis*, *prevotellamelaninogenica*, *streptococcus mitis* infection, it was meaningful difference between two groups ($p<0.01$), in that, 80% of positive subjects went to cancerous event (sensitivity) whereas in 83% of control group (specificity), non of these bacteria were not observed in that, the sensitivity and specificity in parallel groups were 80% and 82% and now it has been well known as good predictor for OSCC (Anttila et al., 2003).

In case-control study by Littman and co-workers, on 508 double sample with anti IgA antibody titer, higher than 16, were cut pointed as positive and this criteria was associated with 1.2 time greater risk for lung cancer (Littman et al., 2004).

It is well known that the change in cell surface carbohydrates is one of the malignant transformation

and the cancer is the molecular disease, that shows the changes in glycoconjugates in cell surface (Hakomori 1996). In the study of Xuan and colleagues for microbial dysbiosis in breast cancer, two species, methyl bacterium radiotolerance and sphingomonasyanoikoe is evaluated as causative agents. They detected DNA of these two bacteria on paraffin embedded and fresh frozen breast tissues.

From different kinds of bacteria, they found the meaningful difference for these two bacteria in normal breast tissue and cancerous breast tissues ($p < 0.01$, $p = 0.01$ respectively). In comparison in normal and cancer tissues, the sphingomonasyanoikoe, showed the significant difference in normal tissues ($p = 0.04$) and in the same time, the presence the methyl bacterium radiotolerance, was much more higher in cancer tissues ($p = 0.25$) than other bacteria types, but not higher enough that makes it significant comparing to normal tissues.

In Xuan's study, it is shown that the presence is about 40% of sphingomonasyanoikoe in normal tissue. Also, there was the reverse significant correlation between presence of methyl bacterium radiotolerance and sphingomonasyanoikoe in normal tissues ($p = 0.0$), but not significant in tumor tissues Xuan (Xuan et al., 2014; Shargh et al., 2014). Taken together, these data indicate that microbial DNA involvement is present in the breast tissues and that bacteria adapt with tumor microenvironment, serves the area for finding the exact mechanism and bacterial potential in tumorigenesis. The link between dysbiosis and breast cancer which has potential diagnostic and therapeutic implications must be considered. According to our research work, warrants further study into the role of microbes in breast cancer and would be of great interest.

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