Nipple Aspirate Fluid in Japanese Women

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Abstract: Nipple aspirate fluid (NAF) is collected in the duct-lobular complex and can be extracted by warmth, massage of the breast, and nipple suction. NAF is available from non-lactating women without an invasive procedure and is expected to be used for early detection and risk evaluation for breast cancer. European and American reports on NAF sampling have been published, but there are no data on Japanese women. In addition, there are few studies on the protein peptide composition of NAF, and many unsolved problems regarding the stability and characteristics of protein peptide composition remain. We tried sampling NAF in 128 Japanese women and analyzed the sampling rate along with characteristics of the protein composition using surface enhanced laser desorption ionization time of flight mass spectrometry.

Key-Words: Nipple aspirate fluid, Breast cancer, Japanese, Proteomic analysis, SELDI-TOF-MS

1 Introduction

Breast cancer is the most commonly diagnosed cancer in Japanese women,

affecting approximately 40,000 women annually; 10,000 of these women die from this disease each year(1).

Although mass screening with mammography (MMG) and advances in treatments and screenings have reduced breast cancer mortality, mortality rates from breast cancer in Japan continue to increase.

In Japanese subjects, breast cancer occurs most commonly in women in their 40s, although many patients are also diagnosed in their 30s(1). There is still much controversy about whether mass screening with MMG reduces mortality rates among women in their 40s, and there are no reports about the efficacy of this procedure for women in their 30s. Breast ultrasonography or magnetic resonance imaging can detect early breast cancer that cannot be detected by mammography in young dense breasts, but it is not realistic to use these procedures for mass screening due the large amount of time required for these tests and the costs involved. The establishment of an effective and versatile screening and risk evaluation method for this younger generation of women is a pressing need in Japan.

Since Papanicolaou established cytological criterion of nipple discharge in 1958, nipple discharge has attracted attention for its ability to predict the presence of a lesion in the breast (2). In the 1960s, Sartorius showed that a small amount of secretion is in breasts of non-lactating women and established a procedure for sampling this fluid, called nipple aspirate fluid (NAF), using warmth, massage of the breast, and nipple suction to obtain aspirate from the nipple (3).

Women who produce obtainable amount of NAF with proliferating cells have a breast cancer risk 2.4-2.8 times that of women who do not produce NAF (4).

NAF is available without an invasive procedure and is thought to reflect the internal environment of the duct-lobular unit, so it is expected to contain a protein associated with tumor genesis of breast cancer. Proteomic analyses of NAF became possible with the development of various protein analysis devices in the 2000s and bioinformatics procedure (5-8).

2 Problem Formulation

European and American reports on sampling of NAF have been published, but there are no data on Japanese women; in addition, a low sampling rate among Asian women has been reported (9).

Before we introduce NAF as an efficient mass screening method in Japan, we need to determine an appropriate sampling rate for Japanese women, discover specific markers that detect early breast cancer, and identify high-risk groups. However, to do this, we must first understand the basic information regarding the protein peptide contents of NAF.

In this study, we sampled NAF in Japanese women and analyzed characteristics of the protein peptide contents using surface enhanced laser desorption ionization time of flight mass spectrometry (SELDI-TOF-MS).

3 Problem Solution

3.1 Material and Methods

3.1.1 Nipple aspirate fluid

We sampled NAF from 128 Japanese women between March 2007 and October 2009. Written informed consent was obtained from all patients before sampling, and the study was approved by the ethical committee of Komagome Hospital. All participants confirmed age, parity, family history of breast cancer, history of breast cancer, use of hormonal therapy by using questionnaire.

NAF sampling was performed on bilateral breasts in healthy participants and on the intact breast in past breast cancer patients. Lactating and pregnant women and women with a history of bilateral breast cancer were excluded. The sampling procedure was carried out according to the modified method of Sartorius, as previously reported (3, 10). In brief, (1) all breasts were observed by ultrasonography before sampling NAF; (2) the breast was warmed with a heating pad for 10–20 min; (3) after cleansing the nipple with olive oil to remove the keratin plug, the breast was massaged from the chest wall toward the nipple while a clinical research coordination nurse provided nipple suctioning with a milk pump used by lactating women; and (4) the sample was collected into capillary tubes and the volume was measured using a metric ruler. Cell contents and impurities were excluded by centrifugation and stored at -80°C until analysis.

3.1.2 Sample set

Twenty-four specimens were randomly selected to compare the protein peptide profile in the right and left specimens obtained synchronously from the same participant (12 specimens from 6 participants) and investigate the characteristics of protein peptides in terms of color differences (white, transparent, serous). In addition, 3 specimens from 3 nursing mothers were added to identify a homogenous protein peptide pattern between mother's milk and white NAF.

3.1.3 Surface enhanced laser desorption ionization mass spectrometry analysis

Protein peptide profiling of NAF was performed using the ProteinChip system SELDI (PCS4000) reader (Bio-Rad Labs, Hercules, CA, USA). Various chip chemistries (hydrophobic, anionic, cationic, and metal affinity) were initially evaluated to determine which affinity chemistry provided the best NAF profiles in terms of number and resolution of protein peptide peaks, and the cationic exchanging chip (CM10) was selected for further analysis. The selected samples were analyzed in duplicate. Throughout the assay, arrays were assembled in a 96-well bioprocessor, which was shaken on a platform shaker at 300 rpm.

Arrays were charged twice with 150 ul of 100 mM sodium acetate pH4 for 5 min.

Unfractionated NAF samples diluted at 1:20 in phosphate-buffered saline (PBS) were thawed on ice and randomly applied to the arrays. After a 15-min incubation period in a humid chamber, the arrays were washed twice with binding buffer for 5 min. Following a quick rinse with deionized water, arrays were air-dried. A 50% sinapinic acid (Bio-Rad Labs) solution in 50% acetonitrile (Biosolve, Valkenswaard, The Netherlands)/0.5% trifluoroacetic acid (Merck) was applied twice (1.0 μl) to the arrays as the matrix. Following air-drying, the arrays were analysed using the ProteinChip SELDI reader. For mass accuracy, the instrument was calibrated on each day of measurement with All-in-One peptide standard and All-in-One protein standard (Bio-Rad Labs).

3.1.4 Data analysis

The correlation between NAF sampling and clinical features of participants was examined using the χ^2 test calculated using JMP® software (version 7, SAS Institute, Cary, NC, USA). A p value <0.05 was considered statistically significant.

Peak normalization, aligning spectra, and cluster analysis were performed using Ciphergen Express (Bio-Rad Labs, Hercules, CA, USA) using the following settings: first pass, 5.0 S/N, 5.0 valley depth, minimum peak threshold 20%; second pass, 2.0 S/N, 2.0 valley depth.

3.2 Results

3.2.1 Sampling data

We included 128 women with a median age of 40 years (range, 21 – 75 years). The median sample amount was 4 μl (range, 0.5 – 88 μl). NAF was obtained from bilateral breasts in 21 participants and from unilateral breast in 42 participants (right only, 25; left only, 17). The sampling rate of participants was 49.2% (63 of 128 participants) and the sampling rate of breasts was 36.2% (84 of 232 breasts). Sample colors were closely divided into white (n = 29), transparent (n = 29), and serous (n = 26) (Table 1).

Age	
Median,(y)	40
Range,(y)	21-75
Sampling rate of participants	
Collected, n (%)	63 (49.2%)
Not collected, n (%)	65 (50.8%)
Sampling rate of breast	
Collected, n (%)	84 (36.2%)
Not collected, n (%)	148 (63.8%)
Sample amount	
Median, (μl)	5
Range, (μl)	0.5-88
Sample color	
White	29
Transparent	29
Serous	26

Table 1. Nipple aspirate fluid sampling data (n = 128 participants and 232 breasts)

Variable	NAF collection+	NAF collection-	p

Age (years)	<30	14	24	0.40
	≥30, <40	34	45	
	≥40, <50	26	60	
	≥50	10	19	
Parity	Parous	40	67	0.73
	Nonparous	44	81	
Familial history	High risk	10	27	0.20
	Low risk	74	121	
Ultrasonography findings	Positive	32	52	0.08
(cyst or duct ectasia)	Negative	40	108	
Breast cancer history	Positive	7	17	0.44
	Negative	77	131	

Table 2. Correlation between nipple aspirate fluid (NAF) sampling and clinical features

There was no statistical correlation between NAF collection and age, parity, familial history of breast cancer**,** and breast cancer history. Participants who had cyst or duct ectasia demonstrated by ultrasonography were more likely to yield NAF ($p = 0.08$) (Table 2).

3.2.2 Protein and peptide analysis

Details of the protein and peptide analysis for the 24 samples are shown in Table 3. Each specimen had 14 to 48 peaks between 1000 and 100000 Da.

The difference in protein peptide profiles between individuals was significant; however, but in specimens obtained synchronously from bilateral breasts in 4 participants, protein peptide peaks were in agreement 85.7% to 92.9% of the time (Fig.1).

Cluster analysis showed that protein peptide peaks in white NAF were homogeneous and had a character similar to mother's milk even in elderly and nonparous women (Figs. 2 and 3).

No	Participant	Age	Laterality	Color
1	A	35	Right	Serous

2	A	35	Left	Serous
3	B	37	Right	White
4	B	37	Left	White
5	C	43	Right	White
6	C	43	Left	Transparent
7	D	43	Right	Serous
8	D	43	Left	Serous
9	E	31	Right	White
10	E	31	Left	White
11	F	44	Left	Transparent
12	F	44	Right	Transparent
13	G	65	Right	White
14	H	25	Right	Serous
15	I	42	Left	Transparent
16	J	48	Right	Transparent
17	K	43	Left	Transparent
18	L	24	Left	White
19	M	46	Left	Serous
20	N	43	Left	Serous
21	O	44	Right	Serous
22	P	26	Right	White
23	Q	28	Right	Serous
24	R	66	Right	White

Table 3. Details of sample set for protein peptide profiling

3.3 Discussion

The success rate of obtaining NAF in American and European women varies from 42% to 99% (4, 11-17), and several factors have been reported to increase the likelihood of NAF collection, including being aged 35 to 50 years (17) and having a history of childbearing and lactation (9). Nevertheless, we did not find any significant correlations between clinical features and success in obtaining NAF in this study. Success rates of 49.2% in Japanese women, 33% in Chinese women born in Asia (13), and 98% in Kenyan women (15) indicate that there are ethnic differences in the likelihood of sampling NAF, and the rate appears to be lower in Asian women than in non-Asian women. In this study, the sampling rate in women with a history of breast cancer was lower than that in women without a history of breast cancer (29% vs. 37%); however, 9 of 17 non-NAF-yielding participants with a history of breast cancer were receiving hormonal therapy (tamoxifen, aromatase inhibitor, LH-RH analog), which might have influenced the on sampling rate. The mean yields of NAF in American and European women have been reported to range from 10-50 μl (15, 18). In this study, the median amount was 5 μl, suggesting that the NAF yield in Japanese women is lower than that in non-Asian women and it needs to make a special care to handle a very small amount of specimens on NAF study in Japan.

Women who produce NAF are at higher risk of developing breast cancer (4, 19, 20). The fact that many kinds of protein have been identified in NAF suggests that NAF contains a more suitable fluid for detection of activity in the breast than serum, as the proteins in NAF are specific to breast tissue (15, 21). When we have a small specimen for

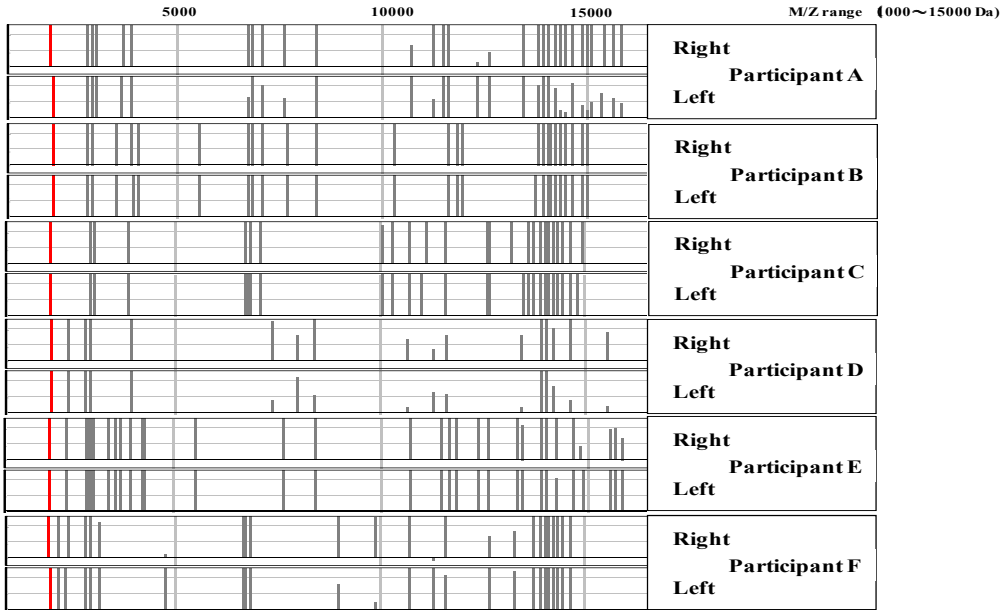

Fig.1 Protein peptide profile in the right and left specimen obtained synchronously from the same participant

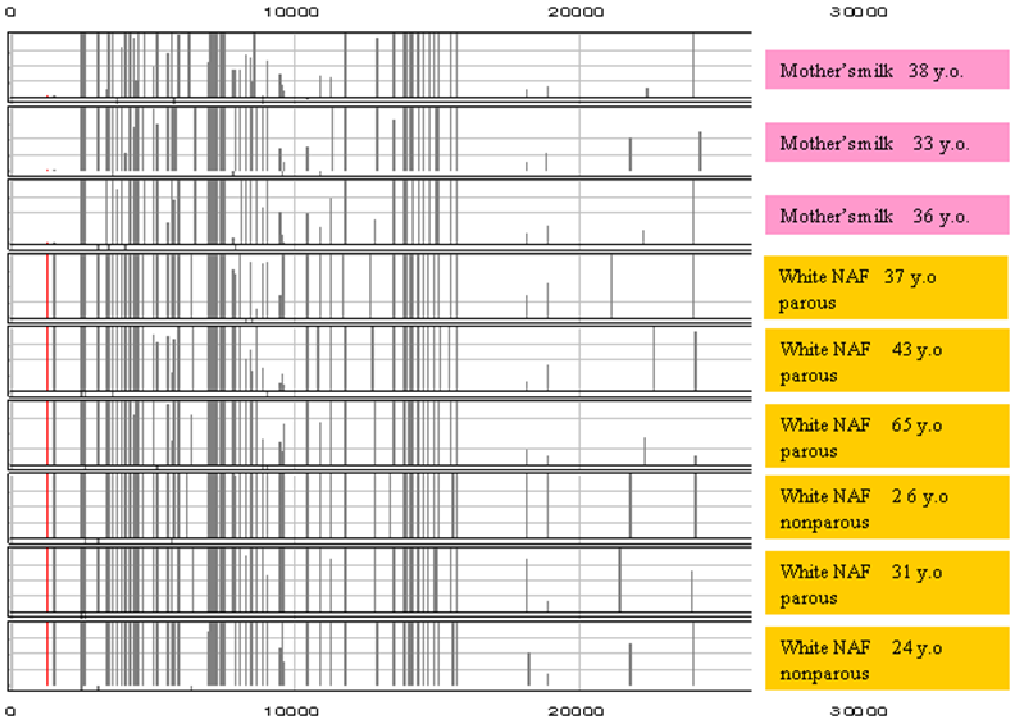

Fig.3 Homogenous protein peptide pattern between mother's milk and white nipple aspirate fluid (NAF)

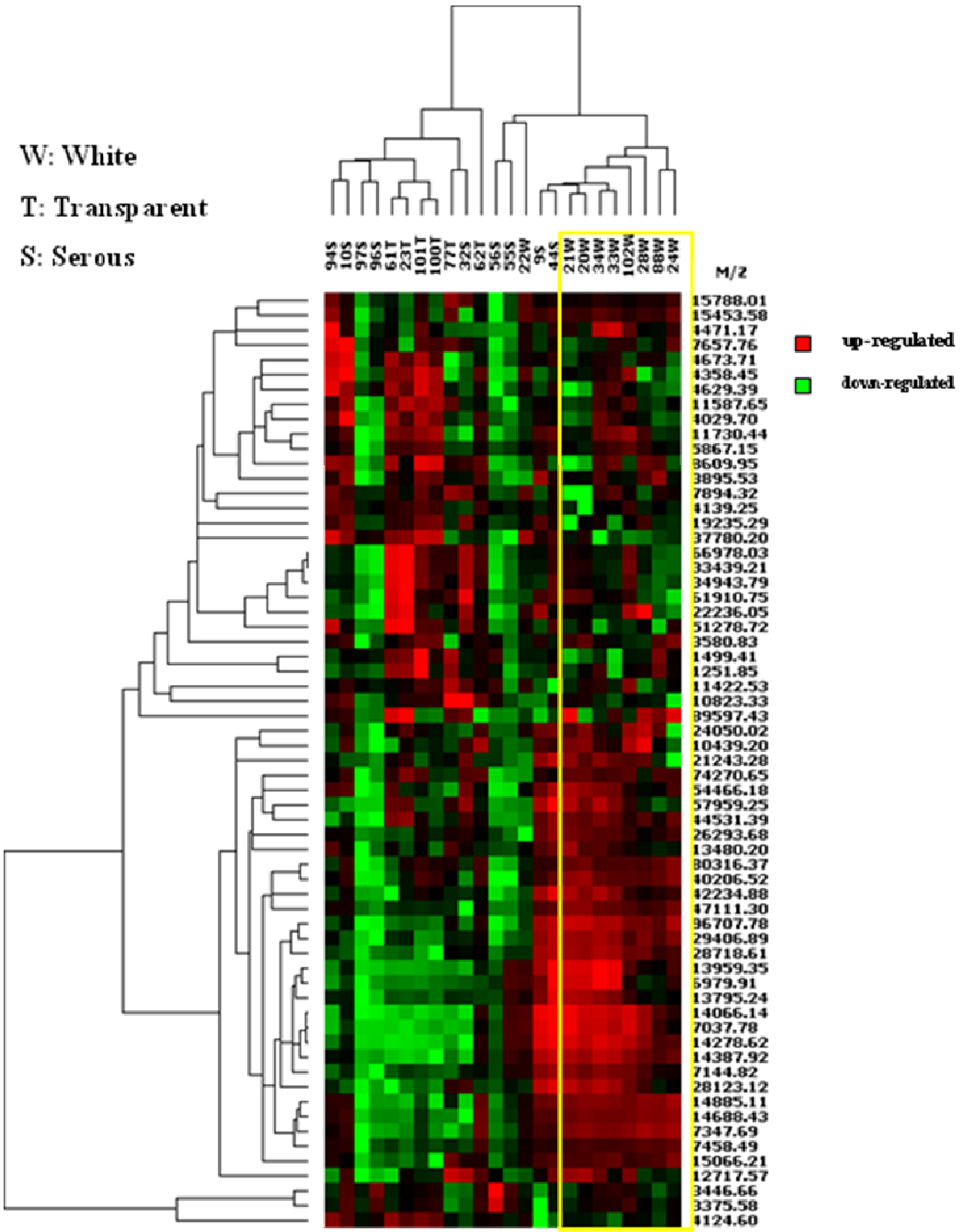

Fig.2 Clustering Analysis

proteomic analysis, SELDI-TOF-MS has several advantages: it easily deals with crude samples, it only needs a small sample, many types of application chip chemistry are available, and multiple software programs for analysis are also available.

Results of the cluster analysis showed that protein peptide profiles differ considerably among individuals, but in specimens obtained synchronously from bilateral breasts from the same participant, the profile is similar. This fact indicates that NAF is likely secreted by a

constant cause and maintains stable composition that reflects the internal environment of the breast in each individual. It also supports the idea that it may be possible to use a NAF sample from only one side when using a screening test to evaluate the risk for breast cancer. In this study, white NAF formed a high homologous cluster with a similar profile to that of mother's milk, suggesting that white NAF is a functional secretion not affected by menopausal status or childbirth history. Thus, it may be necessary to analyze white NAF and non-white NAF separately.

Identification of intrinsic subtypes of breast cancer using clustering analysis in the micro array of complementary deoxyribonucleic acid has been demonstrated to be significantly associated with clinical phenotypes, and the subtypes have been proved to be useful for risk prediction (22). It is possible that clinical phenotypes are regulated by the interaction of several proteins, and we propose that cluster analysis of NAF might lead to the construction of an early detection and risk evaluation tool for breast cancer.

4. Conclusion

We obtained NAF in approximately half the Japanese women in our study and found the specimens to be useful for protein and peptide analysis.

However, numerous proteins and peptides are included in NAF, and there are substantial differences among individuals. Future studies need to focus on the interactions of the different proteins to determine if they can reveal what may happen in the breast at a later time.

We will continue to study additional cases to investigate the specific association of NAF with tumor genesis and identify women with a high risk of breast cancer in the future.

References

[1] Cancer statistics in Japan [database on the Internet] 2008. Available from: http://ganjoho.ncc.go.jp/public/statistics/backnumber/2008_en.html.

[2] Papanicolaou GN, Holmquist DG, Bader GM, Falk EA. Exioliative cytology of the human mammary gland and its value in the diagnosis of cancer and other diseases of the breast. Cancer;11, 64(2),1958, Mar-Apr,pp.377-409.

[3] Sartorius OW, Smith HS, Morris P, Benedict D, Friesen L. Cytologic evaluation of breast fluid in the detection of breast disease. J Natl Cancer Inst;59,65(4),1977, Oct,pp.1073-80.

[4] Wrensch MR, Petrakis NL, Miike R, King EB, Chew K, Neuhaus J, et al. Breast cancer risk in women with abnormal cytology in nipple aspirates of breast fluid. J Natl Cancer Inst; 93, 72(23), 2001, Dec 5, pp.1791-8.

[5] Subhash C.Basak G, Frank Witzmann. Information-Theoretic Biodescriptors for Proteomics Maps: Development and Applications in Predictive Toxicology 9th WSEAS CSCC Multiconference952005.

[6] Nafati. M SM, Rossi. B. Objective data

reduction algorithm of proteomic mass spectrum. Proceedings of the 5th WSEAS int conf on signal processing, Computational Geometry & Aritificial Vasion932005,pp.5.

[7] Nafati. M SM, Rossi. B. A simple and effective detection technique of 2D electrophoresis image protein spots. Proceedings of the 5th WSEAS Int Conf on Signal Processing, Computational Geometry & Artificial Vision942005,pp.5.

[8] Terzidis SPK. Classification Process Analysis of Bioinformatics Data With A Support Vector Fuzzy Inference System. Proceedings of the 8th WSEAS International Conference on Neural Networks962007,pp.6.

[9] Wrensch MR, Petrakis NL, Gruenke LD, Ernster VL, Miike R, King EB, et al. Factors associated with obtaining nipple aspirate fluid: analysis of 1428 women and literature review. Breast Cancer Res Treat;15,75(1),1990, Jan,pp.39-51.

[10] Ohtsuka T, Aruga T. Nipple aspirate fluid in Japanese women. Japanese J of Breast Cancer;23,78(6),2008,pp.562-62.

[11] Chatterton RT, Jr., Geiger AS, Khan SA, Helenowski IB, Jovanovic BD, Gann PH. Variation in estradiol, estradiol precursors, and estrogen-related products in nipple aspirate fluid from normal premenopausal women. Cancer Epidemiol Biomarkers Prev;13,81(6), 2004, Jun,pp.928-35.

[12] Dooley WC, Ljung BM, Veronesi U, Cazzaniga M, Elledge RM, O'Shaughnessy JA, et al. Ductal lavage for detection of cellular atypia in women at high risk for breast cancer. J Natl Cancer Inst;93,67(21),2001, Nov 7,pp.1624-32.

[13] Petrakis NL, Lee MM, Wrensch MR, Ernster VL, Miike R, Koo LC, et al. Birthplace and yield of nipple aspirate fluid in Chinese women. Cancer Epidemiol Biomarkers Prev;7,79(9),1998, Sep,pp.835-9.

[14] Rose DP, Lahti H, Laakso K, Kettunen K, Wynder EL. Serum and breast duct fluid prolactin and estrogen levels in healthy Finnish and American women and patients with fibrocystic disease. Cancer;57,85(8),1986, Apr 15,pp.1550-4.

[15] Varnum SM, Covington CC, Woodbury RL, Petritis K, Kangas LJ, Abdullah MS, et al. Proteomic characterization of nipple aspirate fluid: identification of potential biomarkers of breast cancer. Breast Cancer Res Treat;80,82(1),2003, Jul,pp.87-97.

[16] Wynder EL. Dietary habits and cancer epidemiology. Cancer;43,69(5 Suppl),1979, May,pp.1955-61.

[17] Wynder EL, Lahti H, Laakso K, Cheng SL, DeBevoise S, Rose DP. Nipple aspirates of breast fluid and the epidemiology of breast disease. Cancer;56,83(6),1985, Sep 15,pp.1473-8.

[18] Petrakis NL, Barnes S, King EB, Lowenstein J, Wiencke J, Lee MM, et al. Stimulatory influence of soy protein

isolate on breast secretion in pre- and postmenopausal women. Cancer Epidemiol Biomarkers Prev;5,86(10), 1996, Oct,pp.785-94.

[19] Tice JA, Miike R, Adduci K, Petrakis NL, King E, Wrensch MR. Nipple aspirate fluid cytology and the Gail model for breast cancer risk assessment in a screening population. Cancer Epidemiol Biomarkers Prev;14,89(2),2005, Feb,pp.324-8.

[20] Wrensch MR, Petrakis NL, King EB, Miike R, Mason L, Chew KL, et al. Breast cancer incidence in women with abnormal cytology in nipple aspirates of breast fluid. Am J Epidemiol;135,88(2), 1992, Jan 15,pp.130-41.

[21] Alexander H, Stegner AL, Wagner-Mann C, Du Bois GC, Alexander S, Sauter ER. Proteomic analysis to identify breast cancer biomarkers in nipple aspirate fluid. Clin Cancer Res;10,61(22),2004, Nov 15,pp.7500-10.

[22] Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol;27,90(8),2009, Mar 10,pp.1160-7.