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Cytogenetic Damage in Thyroid Solitary Hypo-Functioning Nodule Patients and Presence of Thyroid Cancer

KEYWORDS: Micronuclei, Thyroid cancer, Solitary thyroid nodule, DNA damage

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ABSTRACT *Aim :To observe the extent of cytogenetic damage in the peripheral blood lymphocytes (PBLs) using micronuclei (MN) frequency in solitary hypo-functioning thyroid nodule patients as an indicator and its possible association with the presence of thyroid cancer.*

Methods: Thirty one patients with solitary hypo-functioning thyroid nodule underwent fine needle aspiration cytology (FNAC), Technetium-99m-pertechnetate (99mTcO4–) scan, and other laboratory tests for evaluation of the nodule status. MN frequency in PBLs in these patients was studied using cytokinesis-blocked assay.

Results: Out of 31 patients with solitary hypo-functioning thyroid nodule, 10 turned out to be thyroid cancer. However, only 7 patients from thyroid cancer group had shown FNAC positive results before surgery. MN frequency demonstrated significant elevation in all the 10 thyroid cancer patients in comparison to non cancer group (61.1 ± 32.3 MN/1000 BN cells vs 25.6 ± 17.0 MN/1000BN cells, P = 0.001). In addition, distribution of more than one MN in binucleated PBLs was higher in thyroid cancer patients than their non cancer counterpart.

Conclusion: The present study indicates significant elevation in DNA damage represented by increased PBL MN frequency in presence of thyroid cancer. It will be interesting to further explore the index of DNA damage as a mass screening marker for estimating risk or early detection of malignancy in thyroid disorder patients.

Introduction

Cancer is known to result from an accumulation of multiple genetic changes which mediates through chromosomal aberrations, and detected cytogenetically.(Solomon 1991) The extent of DNA damage in peripheral blood lymphocytes (PBLs) is known to reflect the amount of genetic damage in the precursor cells that lead to the carcinogenic process in target tissues.(Iarmarcovai et al.,2008a; Norppa et al., 2006; Bonassi et al., 2007; Boffetta et al., 2007; Bonassi et al., 2008) Presence of cytogenetic damage and cancer incidence is being explored since long time and numerous reports have shown positive correlation of cytogenetic damage in PBLs with cancer risk in patient population. (Norppa et al., 2006; Bonassi et al., 2007; Boffetta et al., 2007; Bonassi et al., 2008; El-zein et al., 2006; Iarmarcovai et al., 2008b; Pathak et al., 1982; Sigurdson et al., 2005; Violot et al., 2005; Joseph et al., 2009; Maeffi et al., 2014) However reports on linking thyroid cancer and presence of DNA damage are limited . (Pathak et al., 1982; Sigurdson et al., 2005; Violot et al., 2005; Joseph et al., 2009; Kinashi et al., 2007) It has been observed that amongst various thyroid disorders such as hypothyroidism, hyperthyroidism and presence of the solitary hypo-functioning thyroid nodule represents a form of benign tumor and occurs in approximately 8 % of the general population.(Mazaferri 1993) It has been associated with relatively high incidence of malignancy. In such cases a detailed examination of the solitary thyroid nodule using ultra sonography, nuclear medicine techniques as well as FNAC for checking the presence of thyroid cancer is recommended by the physician. (Mazaferri 1993,; Yeung et al., 2008) These patients are kept under monitoring as they are considered to be at the higher risk for presence of cancer (Mazaferri 1993; Yeung et al., 2008; Mazaferri 1992.) As cytogenetic changes have been known to accumulate in cancerous state it was interesting to observe the same in PBLs of solitary cold nodule patients and explore its association with presence of thyroid cancer .

Volume : 6 | Issue : 5 | May 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

Among several cytogenetic tests, the cytokinesis-blocked MN assay in PBLs is extensively used to study the extent of DNA damage in human population. (El-Zein et al.,2006; Iarmarcovai et al., 2008b; Fenech et al., 1999) Due to its simplicity and reliablility its been highly recommended technique for cancer mass screening programmes measuring the presence of DNA damage in blood samples.(Maffei et al., 2014) The detailed prospective analysis on a large international cohort of subjects has assessed the usefulness of MN frequency in PBLs as a possible predictive cytogenetic marker of cancer risk majorly in urogenital and gastrointestinal cancers .(Bonassi et al., 2007; 2000) We carried out a preliminary study for assessing the DNA damage by measuring the MN frequency in PBLs of solitary hypo-functioning thyroid nodules patients for exploring its association with the presence of thyroid cancer.

Materials and methods

Patients

Thirty one patients with solitary thyroid nodules included in this study visited our institute for their thyroid check-up. Regular thyroid function tests were performed using blood samples from these patients at the first visit. In addition Technetium-99m-pertechnetate ($^{99m}TcO_4^-$) scan was done to assess the functional status of the thyroid nodule. $^{99m}TcO_4^-$ scan was performed by intravenous (i.v.) injection of 3-5 mCi of activity 30 minutes prior to the neck scan using Siemens gamma camera (e.cam). Ultrasonography (USG) was done to confirm the presence of a single nodule and only those patients who showed a solitary nonfunctioning thyroid nodule were included in this study. USG characteristics of benignity or malignancy of a solitary cold thyroid nodule were not considered in this study. The study was approved by the Institutional Ethics Committee. Informed consent was obtained from all the patients before collection of the blood samples. All the patients were checked for the possibility of any other disease such as diabetes, hypertension, cardiac and kidney disease with the history of the medical treatment, if any, at the time of blood collection.

All the 31 patients were studied for the MN frequency in PBLs before their final diagnosis. Out of these 31 patients, 28 underwent FNAC of the thyroid nodule. Amongst these 28 patients 10 underwent thyroidectomy due to the presence cancer on FNAC or presence of follicular neoplasm or large goiter size. The final histopathology report (HPR) obtained for all these 10 patients confirmed presence of thyroid cancer.

The remaining 21 patients were benign on FNAC and were followed up clinically for 3 years and none of these patients showed an increase in the size of the nodule or a morphologic change on ultrasonography. These patients are still on follow up.

On the basis of histopathological evidence of presence or absence of cancer we have classified the total number patients in two groups Gr I: Non-cancer group (n=21) and Gr II: Cancer group (n=10). We have compared MN frequency in these two groups and have tried to see its implication in presence or absence of thyroid cancer.(Fig.1)

Micronuclei Analysis

Heparinized blood was collected from all the 31 patients for detection of MN frequency in PBLs. MN assay was performed by cytokinesis-blocked technique on patients blood as described earlier.(Joseph et al., 2009) Briefly, whole blood cultures in duplicate were set up by using 0.5 ml of heparinized blood to 4.5 ml of Iscove's Modified Dulbecco's Medium (Gibco, USA), supplemented with 10% heat-inactivated foetal calf serum. 40 µg/ml phytohaemagglutinin (PHA-M, Sigma Chem. Co, USA) was added to stimulate the lymphocytes. Cytokinesis was blocked using 6 µg/ml cytochalasin-B (Sigma Chem. Co., USA) in dimethyl sulphoxide after 44 hr of the initiation of the culture. At 72 hr, the cultures were harvested by re-suspending the cells in hypotonic solution (0.8 % potassium chloride). The cells were incubated with fresh and cold Carnoy's fixative (methanol : acetic acid, 3:1). After three washes with fixative, the cells were dropped on to the microscopic slides, air-dried and stained with Giemsa (Sigma Chem. Co.,USA). MN frequency was measured by scoring minimum 1000 binucleate (BN) cells for the analysis. The presence of more than one MN / BN PBLs was also scored and analysed separately.

Statistical analysis

The results are expressed as mean ± standard deviation (S.D.). The mean MN frequency in PBLs of the solitary nonfunctioning nodule patients were compared for their gender, FNAC outcome and the final diagnosis of the patient using the Student's *t*-test. The difference was considered significant at $P < 0.05$. Correlation between the age and MN frequency was tested by using Pearson Correlation Coefficient.

Results

On the basis of histopathological report of presence or absence of cancer we have classified the total 31 patients in two groups Gr I: Cancer (n=10) and Gr II: Non-cancer group (n=21). Fig.1

In Gr I all the 10 patients were diagnosed of thyroid cancer on the basis of their HPR post surgery (Table 1) (Fig.1). In these 10 thyroid cancer patients, 7 were FNAC positive for cancer while one was benign (patient no. 7) and in two (patient no. 9 and 10), it was follicular neoplasm. However, due to a large goiter size, the patient no. 7 was operated after 3 months of initial investigations and found to have papillary thyroid cancer with focal follicular differentiation alongwith regional lymph node metastasis on HPR. Rest of the two patients showing follicular neoplasm (patient no. 9 and 10) were also operated at 72 and 85 days respectively after the FNAC test and found to have follicular cancer of thyroid with lymph node metastasis and differentiated papillary thyroid cancer having extra thyroidal extension respectively.

In rest of the 21 patients presence of solitary non functioning thyroid nodule was demonstrated on FNAC, $^{99m}TcO_4^-$ scan and USG without any detectable malignancy (Table 2) (Fig.1). Out of these 21 patients, 18 were subjected to FNAC of thyroid nodule and 3 patients showed non-diagnostic FNAC results. In this group 4 patients were suffering from thyroidal and non-thyroidal disease such as acute abscess (patient no. 3), hyperthyroidism (patient no. 4), mitral stenosis (patient no. 5) and bronchial asthma (patient no. 11) and were on medication.

When MN frequency was measured in PBLs of all the patients, a significant increase in MN frequency was observed in Gr I consisting of thyroid cancer patients when compared with the Gr II of non cancer patients (P=0.001,Table 3). Significant increase was also observed in MN frequency when it was compared among FNAC

Volume : 6 | Issue : 5 | May 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

positive and FNAC negative patients of the study group (P=0.001).

Mean age of the cancer and non cancer group did not show statistically significant change (46.9 ± 10.29 yrs vs 38.1 ± 12.86yrs, P=0.069) However when possible influence of age on MN frequency was studied in all the 31 patients irrespective of their disease status, showed positive correlation (r ˷ 0.53, P< 0.01). No statistically significant difference was observed in the MN frequency when compared for gender of the study group (P=0.11 Table 3).

The incidence of one MN per binucleated cells in cancer group, was 4.97% as compared to 2.37% in non cancer group. Similarly, cells with two and three MN were 0.43% and 0.08% in thyroid cancer patients when compared to 0.08% and 0.01% in non cancer group. (Table 4.)

Discussion

A cold solitary thyroid nodule is a hypo-functioning nodule in the thyroid gland with an incidence of cancer varying between 8% to 20%. (Yeung et al., 2008; Mistry et al., 2011) It was of interest to observe the accumulation of genetic damage, if any, in the PBLs of such patient group, where the probable risk of thyroid cancer is relatively high. Therefore the present preliminary study was designed to evaluate the MN frequency in PBLs in patients suffering from cold solitary thyroid nodule. FNAC of the thyroid nodule is a gold standard to detect the presence of malignancy (Mazaferri et al.,1993; Carling et al., 2005.) However, it suffers from few drawbacks such as requirement of skilled personnel and the difficulty in differentiating between the follicular adenoma from follicular carcinoma (Carling et al., 2005) Similar was the observation in the present preliminary study wherein among the 10 patients of thyroid cancer, FNAC did not demonstrate the presence of cancer in 3 patients. (Patient no.7, 9 and 10,Table 1).These patients were diagnosed postsurgically as follicular thyroid cancer (patient no.9, Table 1) and of papillary thyroid cancer (patient no.7 and 10, Table 1). Overall increased MN frequency was observed in FNAC positive group as compared with the FNAC negative group in total study population (P=0.001). However all 10 cancer patients showed increased MN frequency irrespective of their pre surgical confirmation of cancer by FNAC. This indicates the increased DNA damage in the PBLs in presence of thyroid cancer which may prove to be valuable while denoting the early cytogenetic changes in thyroid cancer.

Formation of nuclear anomalies such as chromosomal rearrangement and breakage which may give rise to MN are early events commonly observed in the initial stages of carcinogenesis (Umegaki et al., 2004; Norppa 2004) On the basis of possibility of increased cytogenetic damage, which could reflect an enhanced cancer risk, various cytogenetic end points have been used as biomarkers of malignancy detection.(Iarmarcovai 2008b ; Gisselson & Hoglund 2005., Hagmar et al.,1998) Several studies have shown positive correlation of chromosomal aberration frequency in PBLs with the prediction of increased cancer risk.(Bonassi et al., 2008;Norppa 2004; Rudolph et al., 2001; Stewenius et al., 2005) Among several cytogenetic end points, MN frequency and its association with risk of cancer development was also considered due to mechanistic similarities with chromosomal aberration which has been considered for cancer prediction.(Norppa et al., 2006; Bonassi et al., 2007; Boffetta et al., 2007; El- Zein et al., 2006; Pathak et al., 1982; Bonassi et al., 2000; Carling et al., 2005) Bonassi et al have reported an extensive study, where healthy subjects were screened for genetic damage and they have found a positive correlation between MN frequency in PBLs and cancer incidence, especially in urogenital and gastrointestinal cancer.(Bonassi et al., 2007) In case of thyroid cancer, limited studies have been done in past, exploring the association of thyroid cancer with chromosomal damage.(Pathak et al., 1982 ;Joseph et al., 2009; Kinashi et al., 2007)Recently, presence of genetic mutations such as B type Raf kinase (BRAF) mutations in thyroid biopsy samples are being explored for its utility as a diagnostic marker for papillary thyroid carcinoma.(Xing et al., 2009) However these specialized investigations turn out to be expensive and not yet included in the regular investigation panel of tests for thyroid cancer .

In the present study we have observed significant elevation in MN frequency in those 10 patients in whom thyroid cancer was confirmed on HPR in comparison to rest of the non-functioning nodule patients. Various investigators have observed significant elevation in MN frequency in patients of different type of cancers even before the initiation of their treatment such as chemotherapy or radiotherapy .(Venkatachalam et al.,1999; Fenech et al., 1990; Jagetia et al., 2001) Most of the cancer patients in our study showed significantly increased MN frequency of two or more MN in bi nucleate cell in comparison to patients without malignancy (Table 4). It is possible that MN frequency started increasing after the initiation of the disease and could be a consequence of the disease condition. Similar changes have been observed in past in case breast, pharynx and uterus cancer. (Jagetia et al.,2001; Milosevic et al. 2010) Maffei et al in their study have included measurement of PBL MN frequency along with other tests in the screening programme of colorectal cancer and observed significant increase in the PBL MN frequency in the group of patients exhibiting presence of colorectal cancer when compared to the patients showing presence of colorectal polyp and in other participants of the screening programme who were disease free. The present study also demonstrates increase in MN index in thyroid cancer patients when compared with non cancerous solitary non-functioning nodule patients (25.6 ± 17.0 MN/1000 BN cells, n =21) and normal healthy control subjects studied in our laboratory in past (7.8 ± 3.4 MN/1000BN cells, n=38).(Joseph et al. 2009.) Presence of nonfunctioning nodule represents 10-15% higher probability of risk of thyroidal malignancy and therefore, these patients are investigated further for possible occurrence of cancer by conventional nuclear medicine techniques. The higher MN incidence in present patient group seems to be an interesting observation indicating increased genetic instability in PBLs of patients diagnosed with thyroid cancer.

MN frequency is also known to increase in various diseased conditions, medication and aging. In the present study group, four patients were found to have other health problem in addition to non-functioning thyroid nodule and were also on medication (patient no.3,4,5,and 11). These four patients demonstrated higher MN frequency in comparison to remaining 17 solitary nonfunctioning nodule patients (Table 2). Age has also been reported to influence the frequency of MN in PBLs of humans.(Fenech 1986) We have also observed a positive correlation of MN frequency with aging in the total study group. However age may not be the confounding factor for observed raise in MN frequency in cancer group as compared with the non can-

Volume : 6 | Issue : 5 | May 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

cer group as mean age of patients in both groups remains comparable (P=0.069).

PBL MN has been proposed as a potential candidate for noninvasive, simple and reliable cancer biomarker for estimating relative risk of cancer and for the early detection of cancer.(Bonassi et al. 2007; Maffei et al. 2014) It can serve as a feasible, cost effective option for screening of patients in high risk group of thyroid cancer. The instances where FNAC technique remains inconclusive or even repetition of the technique can not resolve the presence or absence of cancer PBL MN measurements may serve as an important tool for close monitoring of these patients. Solitary hypo-functioning nodule represents a high risk group for the presence and development of thyroid cancer and these patients need to be monitored regularly for observing the presence of cancer. As PBL MN frequency represents notable changes in early phase of cancer development, its worthwhile exploring the utility of MN frequency as a biomarker for cancer risk estimation . This information will be complementary in the follow up of solitary cold nodule patients for thyroid carcinoma in larger study population.

Figure 1.
Total study group (n=31)

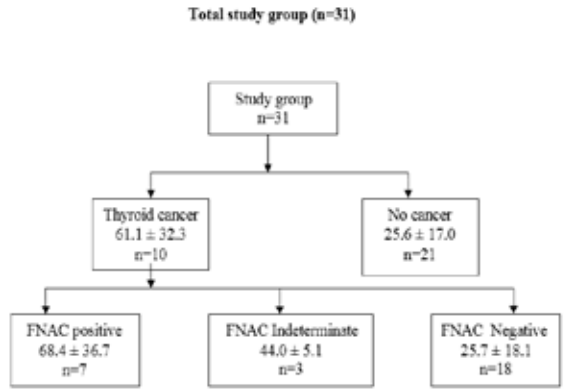

Table 1. Clinical characteristics and micronuclei index in thyroid cancer patients. (n=10)

S. No	Age (Yrs)	Gender	Functional status (thyroid scan)	FNAC	TFT	HPR	MN/1000BN cells
1	55	M	NF	Papillary thyroid cancer.	Normal	Papillary thyroid cancer.	54.3
2	54	M	NF	NHL	Normal	Low grade NHL of thyroid with background Hashimoto's thyroiditis.	107.4
3	53	M	NF	Papillary thyroid cancer.	Normal	Differentiated Follicular variant of Papillary thyroid cancer.	114.3
4	43	F	NF	Thyroid cancer	Normal AMA mildly raised	Hurthle cell variant of Papillary thyroid cancer. (surrounding areas show Hashimoto's thyroiditis.)	45.3
5	29	F	NF	Papillary thyroid cancer.	Normal	Papillary thyroid cancer.	30.5
6	61	M	NF	Atypical cells	Normal	Follicular thyroid cancer	96.9
7	42	M	NF	Benign lesion	Normal	Differentiated Papillary thyroid cancer with focal follicular differentiation with regional LN mets	39.7
8	32	F	NF	Metastatic Papillary thyroid cancer.	Normal AMA raised	Differentiated Papillary thyroid cancer with LN mets and ETE	30.0
9	50	F	NF	Follicular neoplasm	Normal	Follicular thyroid cancer with LN mets	42.7
10	50	F	NF	Follicular neoplasm	Normal	Differentiated Papillary thyroid cancer with ETE	49.6

FNAC: Fine needle aspiration cytology,TFT: Thyroid function test NF:nonfunctioning,, NHL:non-Hodgkin's lymphoma, LN mets:Lymph node metastasis

Volume : 6 | Issue : 5 | May 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

Table 2. Clinical characteristics and micronuclei index in non cancer thyroid patients. (n=21)

S No	Age (yrs)	Gender	Functional status (Thyroid scan)	FNAC	TFT	Non thyroidal disease	MN/ 1000BN cells
	40	F	NF	Colloid goiter	Normal	No	13.2
	42	F	NF	Non-diagnostic.	Thyroiditis AMA raised	No	29.1
	38	F	NF	Acute Abscess	Normal	Acute Abscess	75.3
	50	F	NF	ND	Hyperthyroid	No	52.1
5	36	F	NF	Nodular goiter-Benign	Normal	Mitral Stenosis	34.6
6	43	F	NF	Benign follicular lesion	Normal	No	15.2
7	25	F	NF	Colloid goiter	Normal	No	25.8
8	43	F	NF	ND	Normal	No	21.1
9	30	F	NF	ND	Normal	No	36.5
10	40	F	NF	Colloid goiter	Normal	No	35.8
11	65	F	NF	Non- diagnostic.	Normal	Bronchial. Asthma	45.7
12	31	F	NF	Colloid nodule	Normal	No	20.1
13	21	F	NF	Colloid nodule	Normal	No	21.0
14	14	F	NF	Benign nodule of thyroid	Normal	No	13.5
15	34	F	NF	Colloid nodule	Normal	No	13.5
16	65	F	NF	Colloid goiter	Normal	No	29.1
17	31	M	NF	Non-diagnostic	Normal	No	22.5
18	45	F	NF	Colloid goiter	Normal	No	3.5
19	27	F	NF	Benign thyroid goiter	Normal	No	10.5
20	29	M	NF	Benign follicular cells and colloid	Normal	No	4.8
21	51	F	NF	Colloid goiter	Normal	No	15.1

FNAC: Fine needle aspiration cytology, NF: nonfunctioning , ND: Not done ,TFT: Thyroid function test

Table 3 Micronuclei frequency in peripheral blood lymphocytes of total study group

Group	Number (n)	MN/1000 BN cells (mean ± SD)	Significance
Gender			
Male	7	62.8 ± 43.5	P=0.110
Female	24	29.5 ± 16.5	
Cancer			
Present	10	61.1 ± 32.3	P=0.001
Absent	21	25.6 ± 17.0	
FNAC			
Positive	7	68.4 ± 36.7	P=0.001
Negative	18	25.7 ± 18.1	

Table 4 Distribution of micronuclei frequency in patient population (n= 31)

Patients	MN/1000BN cells (mean ± SD)	Analysed cells	Distribution of MN			
			1MN	2MN	3MN	4MN
Gr I: Cancer (n=10)	61.1 ± 32.3	10000	497 (4.97%)	43 (0.43%)	8 (0.08%)	1 (0.01%)
Gr II:Non cancer (n=21)	25.6 ± 17.0	21000	498 (2.37%)	17 (0.08%)	2 (0.01%)	0 (0.00)

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Volume : 6 | Issue : 5 | May 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

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