

Transmissible genes induced chromosome-type aberrations in the lymphocytes of multiple endocrine neoplasia type 1 and type 2A (MEN1 and MEN2A) patients

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Abstract

Multiple endocrine neoplasia (MEN1& MEN2) are autosomal dominant disorders presenting tumors in two or more organs such as parathyroid tumors and anterior pituitary glands so on. Lymphocyte chromosomes from a MEN1 patient with an anterior pituitary tumor including her sisters and children and 5 MEN2A patients with medullary thyroid carcinoma were studied for the genetic instability. Chromosome type aberrations such as dicentrics, rings, minutes, translocations and deletions were found in 20% of the observed cells of the MEN1 patient. Three of the 5 patients showed a slight increase in this type of aberrations in 6.4, 4.3 and 1.1% of observed cells. Contrarily, frequencies of spontaneous chromatid type aberration in both MEN1 and MEN2A patients were not significantly higher than of controls. There were no increase in the MTX-induced chromatid breaks and no unique fragile sites were observed in all MEN2A patients. High incidence of chromosome instability (only chromosome type aberrations) in the lymphocytes of MEN1 and MEN2A patients presents an interesting observation to understand the genetic pathology prone to the hereditary type tumor development of acquisition of more advance stage tumors and the mechanisms for how transmissible genes induce chromosome type aberrations alone.

Keywords: Multiple endocrine neoplasia, chromosome instability, medullary thyroid cancer.

Introduction

Multiple endocrine neoplasia (MEN) is defined as a disorder with neoplasm in two or more different hormonal tissues and autosomal, dominantly inherited syndromes (Marx, 2005; Jensen *et al.*, 2008; Tukada *et al.*, 2008). Type 1 and type 2 of multiple endocrine neoplasia (MEN1 & MEN2) are autosomal dominant disorder with predisposes to neoplasia development. Affected MEN1 patient develop tumors or hyperplasia in two or more organs such as parathyroid glands, anterior pituitary and endocrine pancreas. On the other hand, MEN2 patients usually develop medullary thyroid carcinomas and may also develop pheochromocytoma and parathyroid adenomas. *MEN1* gene is at 11q13 of chromosome 11 and it encodes a 61-amino acid protein termed menin. *MEN1* gene is a tumor suppressor gene by several criteria (Byström *et al.*, 1990; Chandrasekharappa *et al.*, 1997). MEN2A and 2B are caused by germinal mutations in the *RET* proto-oncogene mapped at 10q11.2 (Santoro *et al.*, 1995). The protein is a transmembrane receptor tyrosine kinase. Based on clinical symptom, the multiple endocrine neoplasia type 2 (MEN2) is divided into three groups: MEN2A, MEN2B and familial medullary thyroid carcinoma (FMTC). MEN2A is characterized by the association of medullary thyroid carcinoma, pheochromocytomas and hyperparathyroidism, while MEN2B includes medullary thyroid carcinoma, neuromas of the lips, tongue and conjunctivae, intestinal

ganglioneuromatosis, and skeletal abnormalities. The medullary thyroid carcinoma is the sole clinical manifestation in FMTC. In MEN2A, the disease links to point mutation of *RET*, which are mostly located to cysteine residues in the extracellular cysteine-rich domain. These point mutations introduce abnormal intermolecular cysteine bridges, and cause an autoactivation of RET by dimerization. MEN2 subtypes have distinct mutational spectrums in *RET* and vary in severity. The most severe disease subtype, MEN2B, is associated with a specific *RET* mutation of M918T. RET-mediated gene expression pattern is affected by subtype of MEN2 (Hickey *et al.*, 2009).

Increased number of cytogenetic abnormalities in lymphocytes has been reported in both diseases (Sasaki *et al.*, 1980; Gustavson *et al.*, 1983; Babu *et al.*, 1985; Benson *et al.*, 1988; Scappaticci *et al.*, 1991; Hecht & Hecht, 1991). A consistent degree of chromosome instability was found in cultured lymphocytes and fibroblasts derived from skin biopsies of MEN1 patients and MEN2 patients (Hus *et al.*, 1981; Krizman *et al.*, 1987; Le Coniat *et al.*, 1987; Benson *et al.*, 1988; Scappaticci *et al.*, 1991; Tomassetti *et al.*, 1995; Sakurai *et al.*, 1999).

A higher frequency of chromosome aberration might be a predisposing factor for the development of cancer. Specific chromosome analysis involving comparison of frequencies of chromosome type aberrations, chromatid-

type aberrations and methotrexate (MTX) induced chromatid aberration involving common fragile sites, and the observation of breakpoint distribution of chromatid aberrations, are essential for understanding the chromosomal instability in these neoplasias. The above investigations are lacking and the literature on the cytogenetic analysis of MEN1 and MEN2A is very few. Hence, we did the precise analysis of data in lymphocytes of MEN1 and MEN2A patients.

Table 1. Chromosome aberration frequencies in lymphocytes from MEN1 patient & her siblings.

	Exa. cells	Chromosome aberration				Chromatid aberration		
		Ab. cells (%)	Struct. -ab.	Hyper 2n+α	Tetra. 4n	Ab. cells (%)	break	gap
Patient	100	20(20.0)	16	1	3	2(2)	2	0
Elder sister	30	0	0	0	0	3(10)	2	1
Young sister	30	1(3.3)	0	1	0	1(3.3)	1	0
Son	20	0	0	0	0	2(10)	2	0
1 st daughter	20	0	0	0	0	0	0	0
2 nd daughter	20	0	0	0	0	2(10)	2	0
Normal persons (N=2)	200	0(0)	0	0	1	12(6.0)	9	3

Exa.: Examined, Ab: Abnormal, Struct. Ab.: Structural aberration, Hyper: hyperdiploid, Tetra.: Tetraploidy, Ab. cells: Abnormal cells.

Materials and methods

Patients: One MEN1 patient was 58 years old Japanese female and she had tumors of anterior pituitary, accessory thyroid and pancreas. As anterior pituitary secreted ACTH, the MEN1 had an accompanied Cushing's disease. Normal siblings of the MEN1 patient (two sisters, son & first & second daughters) were also analyzed for chromosome aberrations in lymphocytes. All of 5 MEN2A patients (3 males & 2 females, and their average ages was 53 years) were American Caucasus and they were diagnosed as MEN2A. Out of the 5 patients each two patients were siblings as brother and sister, and brothers, respectively. For control, 2 normal healthy age-matched Japanese persons and 6 normal healthy age-matched American Caucasus persons (average age of 51 years, 4 males & 2 females) were selected for the present study.

Chromosome analysis: Approximately 10 ml of peripheral blood from each patient and control including the normal siblings of MEN1 patient were used for chromosome analysis of lymphocytes. None of the patient, controls as siblings of MEN1 patient and control persons had a history of radio and chemotherapy. Buffy coat cells were cultured for 72 h with phytohemagglutinin (PHA) and slides were prepared. The slides were treated with Wright's stain solution for G-banding (Yunis, 1981). Well spread 20 to 100 metaphases were photographed and

analyzed for chromosome and chromatid-type aberrations. For high resolution chromosome banding, synchronization method were performed with methotrexate (MTX), which is a name of drug of aphidicoline, fluorodeoxyuridine (FUdR) and thymidine (TdR) or ethidium bromide (EBr). Blood samples were cultured with MTX and TdR and bromodeoxyuridine (BudR) for 72 to 96 h to detect fragile sites only in MEN2A patients. Chi-square and *t*-tests were used for statistical analysis.

Results

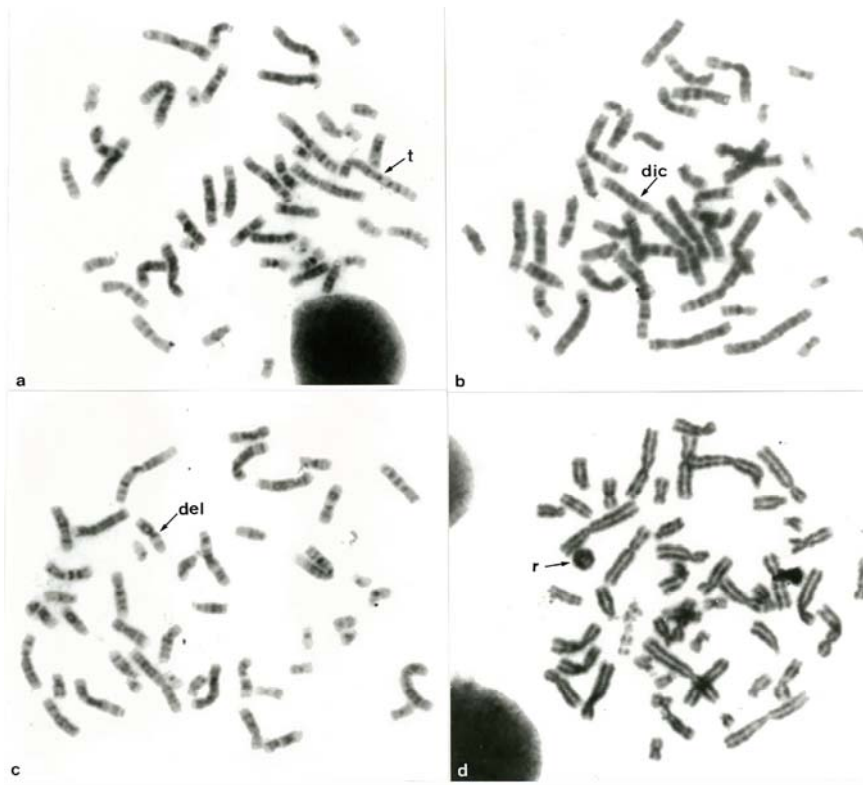
High resolution banding analysis revealed no subtle deletion in all the sets of the chromosome. Putative oncogene associated with MEN1 patients is suggested to be located on chromosome 11 at 11q13 region. However, any rearrangement and deletion were not detected around this region. The chromosomal aberration in lymphocytes of MEN1 patients and her siblings are summarized in Table 1. The lymphocytes of the MEN1 patient showed 20% chromosome-type of aberrations. The proportion was significantly higher than those scored in the two controls, in which the range was from 2 to 4% for chromatid-type aberrations and 0.2 to 0.3% for chromosome-type aberrations (Table 1).

Table 2. Detailed karyotype of abnormal cells found in lymphocytes from a MEN1 patient.

46, XX (normal metaphase)	80 cells
46, XX, -1, +der(1)t(1;?)p22;?)	2 cells
46, XX, t(3;12)(p21;q13)	1cell
46, XXX, -4, +der(4)t(4;?)p?11;?)	2cells
46, XX, del(6)(q21)	1cell
46, XX, del(12)(q13)	1cell
46, XX, -19, t(12;17)(p11.2;q25), +mar	1cell
46, XX, -12, -14, +der(12)t(12;?)q24;?), +der(14)t(14;?)q32;?)	1cell
45, XX, -2, t(2;12)(q11;q24.3)	1cell
46, XX, inv(13)(p11q14)	1cell
47, XXX (hyperdiploid)	1cell
45, XX, -2, dic(2;12)(p25;p13)	2cells
45, XX, -4, +dic(4;10)(p16;q26)	1cell
47, XX, +r	1cell
51, XX, +r, +min, +min, +min, +min	1cell
Tetraploid	3 cells

The MEN1 patient had the highest number of abnormal metaphases; 20 (20%) of the 100 cells examined had numerical and structural abnormalities. Out of the 20 cells, 16 had structural chromosome aberrations such as translocations, deletion and dicentrics which are listed in Table 2. The aberrations of der(1)t(1;?), der(4)t(4;?) and dic(2;12) were observed in 2 metaphases, which indicates clonal proliferation. Various chromosomes were involved in the breakpoints predominantly around telomeric regions. Unstable chromosome aberrations

Fig. 1. G-banded metaphases showing chromosome aberrations in lymphocytes of a MEN1 patient. (a) t: der(1)t(1;?) (p22;?). (b) dic: dic(2;12)(p25;p13). (c) del: del(6)(q21). (d) r: ring chromosome.



such as dicentrics, ring and minute chromosome were found in 5 out of 16 cells. Hyperdiploid and tetraploid metaphases were also found in 3 and 3 cells, respectively. Frequencies of chromatid-type aberrations were 2 percent, which was not higher than that of controls. Four metaphases of MEN1 patient are shown in Fig. 1.

The results of the chromosome analysis in the siblings of the MEN 1 patient are also summarized in Table 1. The 20 to 30 metaphases were analyzed in the lymphocytes of 2 sisters and 3 children did not reveal any chromosome aberrations. One sister had a hyperdiploid cell. Frequencies of chromatid- type of aberrations in these siblings were same as controls. These results imply the MEN1 patient seems to be sporadic case.

Table 3 shows the results of chromosome analysis in 5 MEN2A patients. The frequencies of chromosome and MTX-induced chromatid-type aberrations were 0 to 6% and 8 to 20%, respectively. On the other hand, controls had 13.5% of chromatid-type aberrations induced by

MTX, but no chromosome-type aberrations. Three MEN2A patients (Case nos 1, 2 & 3) had chromosome aberrations presenting inv(14), t(7;14), del(14) and del(6). The aberrations of inv(14) and t(7;14) were observed in 2 metaphases, which indicates clonal proliferation. High resolution banding study on all of two MEN2A patients, who are sisters, showed normal karyotype and did not revealed any subtle abnormalities.

If MEN2A patients had specificity higher fragile sites, the putative oncogene located on the fragile sites might be associated with pathogenesis of MEN2A. Chromatid-type aberrations induced by MTX and BudR were also observed in five MEN2A patients to identify more associated chromosome breakpoints with MEN2A. Treatment of anti-replication chemicals such as aphidicoline, fluorodeoxyuridine (FUdR) also called MTX, bromodeoxyuridine (BrdU) and 5-Azacytidine, which has more clustered distribution of breakpoints on specific fragile sites such as 3p14, 16q23, and 4q21, 9q34.1, and 4q12, 5p13, 5q15, 6q13, 9p21, 10q21, 13q21, and 1q12, 1q42, 9q12, 19q13, respectively (Yunis *et al.*, 1987; Sutherland, 1998). Five different culture

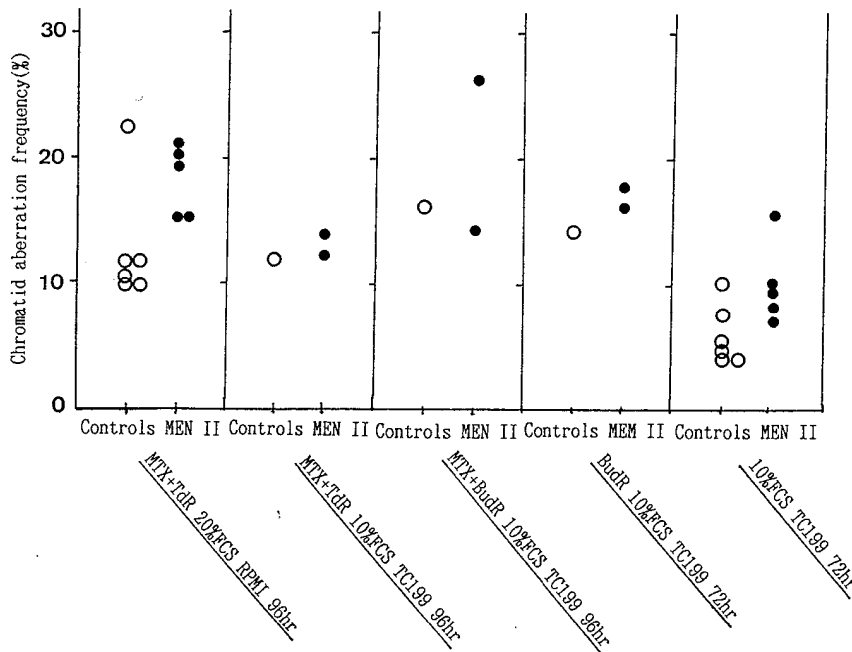
conditions (MTX+TdR in RPMI medium containing 20% FCS for 96h, MTX+TdR in TC199 medium containing 10% FCS, MTX+ BudR in TC199 medium containing 10% FCS for 96 h, BudR alone in Tc199 medium for 72 h and in TC199 medium containing 10% FCS alone for 72 h as a control) were used in this analysis. The frequencies of chromatid-type aberrations induced by MTX and BudR were not

Table 3. Chromosome aberration frequencies found in lymphocytes from 5 MEN2A Patients.

Patient no. MEN2A sex	Analyzed cells	Chromosome aberrations		MTX-induced chromatid aberrations		
		Abnormal cells (%)		Abnormal cells (%)	break	gap
1.F	100	6(6)	inv(14)(q12q34)-2 cells, t(7;14)(p12,q34) - 2 cells del(14)(q12q22), del(?21)(?q22),	19(19)	11	12
2* M	100	1(1)	inv(6)(p12q33)	15(15)	5	8
3* F	100	4(4)	del(6)(q34), del(21)(q21), +21/del(6)(q22), inv(14)(q21q34)	20(20)	5	10
4.# M	50	0(0)		4(8)	2	2
5.# M	50	0(0)		5(10)	2	3
Normal person(N=6)	720	0(0)		97(13.5)	38	51

*, #: Different two siblings, F: Female, M: Male

Fig. 2. Comparison of chromatid aberration frequencies induced by MTX & BudR in MEN2A patients & normal persons.

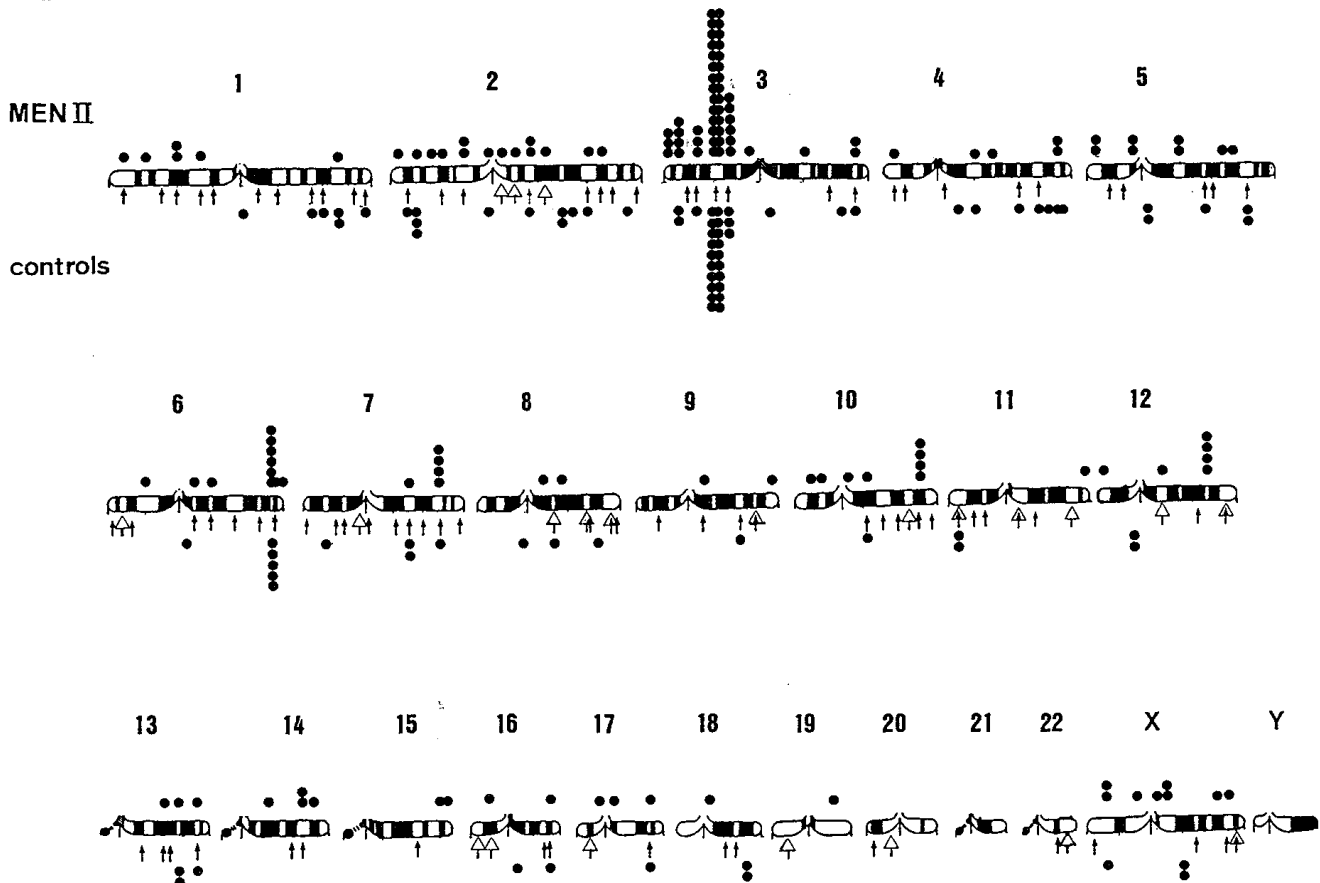


significantly higher than those of controls (Fig. 2). MTX-induced fragile sites in 5 MEN2A patients were compared with those of controls (Fig. 3). Distribution patterns of fragile sites did not show any difference between two populations. Frequencies of fragile sites of 3p21 and 6q24 were higher in both MEN2A patients and also the control group. MEN2A patients did not have any unique breakpoint. Frequency of fragile sites of 10q11 was much lower in five MEN2A patients.

Discussion

Genetic defects arriving from the microscopic changes on chromosomal bands might be responsible for the tumor development in MEN1 and MEN2A. Then, high resolution G-banding analysis was performed on blood specimens of a MEN1 patient and her siblings, and 5 MEN2A patients. Linkage studies in MEN1 and MEN2A, using restriction fragment length polymorphisms (RFLP) sites, have provided the evidence favoring the localization of the

Fig. 3. Comparative distribution of chromatid breakage points, on haploid set of chromosomes, in MEN 2A patients & normal persons. Triangle arrows & small arrows indicate rare fragile sites & common fragile sites regions, respectively





MEN 1 genes chromosome 11 at 11q13 band region and *RET* gene near centromere region of chromosome 10, respectively. Deletions of short arm of chromosome 20 (Babu *et al.*, 1985) and long arm of chromosome 16 (Pfgagner *et al.*, 1990) were reported in thyroid carcinoma of MEN2A patients, but others could not detect (Wurster-Hill *et al.*, 1986; Tanaka *et al.*, 1987). Putative *RET* oncogene was identified on chromosome 10 at 10q11.2 and *MEN1* oncogene on chromosome 11 at 11q13. Our results revealed no congenital chromosomal abnormalities such as deletion in one *MEN1* and all of 5 MEN2A patients studied and there was also no involvement of chromosomes of 10, 11, 16 and 20. Unfortunately, no DNA analyses on *RET* and *MEN1* oncogenes in MEN1 and MEN2A patients, respectively, were performed in present study.

Frequencies of fragile sites of 3p21 and 6q24 were higher in both MEN2A patients and also the control group. Also, no increase in the frequencies of chromatid-type aberrations were observed in both of one MEN1 and five MEN2A patients than the control samples of the present study. Decreased chromosomal instability was also noted in the lymphocyte cultures in terms of chromatid type aberrations including fragile sites treated with MTX, TdR and BrdU of MEN2A patients. Similar results were reported by others. The frequencies of chromatid type of aberrations in MEN1 patients were 3.9% to 7.3% and 1.7% to 10.8% (Gustavson *et al.*, 1983; Le Coniat *et al.*, 1987). These frequencies were observed in normal ranges. No increase of sister chromatid exchanges (SCE) were observed in MEN1 patients (Gustavson *et al.*, 1983). Also, these results are in conflict with those reported frequencies (Krizman *et al.*, 1987; Tomassetti *et al.*, 1995). The reason why there is discrepancy is not clear.

On the other hand, significantly higher incidence of chromosome-type aberrations were found in one MEN1 patient and 3 out of 5 MEN2A patients. The other 2 MEN2A patients did not present high incidence of chromosome-type aberrations. This indicates that the genetic defects in MEN2A patients might not be homogenous. Increase in chromosome-type aberrations and not in chromatid-type aberrations indicate defects associated with DNA repair system and also might be any other gene such as mutator gene. High incidence of chromosome instability (only chromosome type aberrations) in the lymphocytes of MEN1 and MEN2A patients presents an interesting observation to understand the mechanisms for how transmissible genes such as *MEN1* and *RET* induce chromosome-type aberrations alone.

It is widely accepted that unrepaired or misrepaired DNA double strand breaks (DSBs) lead to the formation of chromosome aberrations. DSBs induced by endogenous process associated with oxidative metabolism, or exogenous agents can be repaired either by non-homologous end joining (NHEJ), or homology

directed repair (HDR) in higher eukaryotes. HDR manifested as sister chromatid exchanges (SCE). When cells at late S/G2 phase are irradiated to ionizing radiation, chromatid-type aberrations are produced. In addition, saturation of lesion tolerance by recombination bypass or translesion synthesis may cause block of DNA replication leading to DSBs at stalled replication forks, which result in chromatid-type aberrations. In the chromatid-type aberrations, 113 chromosomal fragile sites have been identified on human chromosomes. Of these, 89 have been termed common fragile sites, i.e., those which are commonly found in all individuals. Recent studies have shown that the cell cycle checkpoint protein ATR and its downstream target genes such as BRAC1, SMC1, CHK1 and FANCD2 are important for maintaining of chromosome stability at common fragile sites (Casper *et al.*, 2002; Arlt *et al.*, 2006; Durkin *et al.*, 2006). Present results on chromosome analyses indicates that transmissible genes, mutated *MEN1* and *RET* cannot activate ATR pathway in carcinogenesis process in MEN1 and MEN2A patients.

An increase of premature centromere division was observed in lymphocyte chromosomes after exposure to alkylating agent in MEN1 patients (Sakurai *et al.*, 1999). *MEN1* gene spans 9.8 kb with 10 exons in the open reading frame. Several additional studies have indicated that menin stabilized the genome and associated with premature centromere division and replication protein A (Sckhodolets *et al.*, 2003). Replication protein A is involved in DNA replication, DNA repair, DNA recombination, and potentially gene transcription. However, it is not clear what role of these menin-interacting proteins play in the development of MEN1. Furthermore, menin localized to chromatin and nuclear matrix, and the association with nuclear matrix is enhanced by gamma rays irradiation. Then, *MEN1* may be involved in the maintenance of chromosome instability (Tomassetti *et al.*, 1995; Itakura *et al.*, 2000). These might be associated with high incidence of chromosome-type aberrations found in present MEN1 patients. In a recent study, menin interacts with FANCD2, a protein involved in a BRCA1-mediated DNA repair pathway and the interaction between menin and FANCD2. Moreover, targeted disruption of *MEN1* results in increased sensitivity to DNA damage (Jin *et al.*, 2003). As an inflammatory reactive oxygen species (ROS) promotes and cooperates with the telomere damage in Fanconi anemia for hematopoietic senescence (Reshef *et al.*, 2008), similar findings might be related to carcinogenesis in MEN1.

In contrast to the findings that most of putative oncogenes isolated from hereditary diseases show recessive phenotype, like *MEN1*, only *RET* oncogene plays as gain of function. *RET* protein expression leads to increased transcription factor HSF1 activation, which correlates with increased expression of stress response genes like HSP70 (Myers & Mulligan, 2004). Similarly,

activation of oncogenic proteins such as RB, fusion BCR-ABL and JAK2 are linked to DNA damage response and chromosome instability, which might be closely related with evolution to aggressive step in neoplasms (Zheng & Lee, 2002; Slupianek *et al.*, 2006; Sallmyr *et al.*, 2008; Plo *et al.*, 2008). Common fragile sites frequently coincide with the location of genes involved in carcinogenesis, although there has been no direct evidence. RET/PTC rearrangement, which are frequently found papillary thyroid carcinoma, were located in common fragile sites FRA10C and FRA10G. These findings suggest that chromatin modification induced by etiological agents might relate to oncogene alteration on chromosome 10q11.2 (Gandhi *et al.*, 2010).

Adenomas developed from *RET* oncogene knockout mice did not have chromosome instability, which indicate that abnormalities of *RET* oncogene is a predisposition of carcinogenesis, and that another modulated oncogenic events will require to convert it to cancer growth (Miscels *et al.*, 1997; Cranston & Ponder, 2003; Scacheri *et al.*, 2004). Loss of heterozygosity (LOH) study on 13 MEN1 patients indicates that MEN1 pancreatic tumors fail to maintain DNA integrity and chromosomal stability, displaying LOH on chromosome 11 in all patients (Hessman *et al.*, 2001). Medullary thyroid tumors developed from MEN2A patients had predominantly LOHs at chromosomes of 1p, 3p25-26, 17p, and 22q (Chung, 1997; Benn *et al.*, 2000; Khosla *et al.*, 1991; Tanaka *et al.*, 1992; Moley *et al.*, 1992) and recently found abnormalities of *p18* gene at 1p32 (van Veelen *et al.*, 2009). In addition, trisomy 10 and loss of *RET* wild allele were implicated recently in tumorigenesis of patients with hereditary papillary renal carcinoma (Huang *et al.*, 2009). TT cell line established from sporadic form of medullary thyroid carcinoma of a MEN2A patient had abnormalities on chromosomes add(1)t(p36), der(3)t(3;14)(?;q27-3p12:: q?12), add(5)(q?33), add(7)(q31), der(8)t(8;24-p22::q13), add(10)(q25), ins(11)(q13), add(12)(p13), der(14)t(4; 11)(p16;?p13), i(1)(q10) and del(20)(q?13). Insertion of chromosome 11q13, where the gene calcitonin locates. They were unable to detect any aberrations of chromosome bands at 10q11.2 and 20p12 (Tanaka *et al.*, 1987). However, comparative genomic hybridization (CGH) analysis revealed gains of chromosomal regions of 1p, 3q26.3-q27, 4, 9q13-q22, 13q, 22q and chromosome 19 in sporadic medullary thyroid carcinoma (Marsh *et al.*, 2003). These abnormal regions were not consistent with the regions of induced fragile sites detected in peripheral blood lymphocytes from 5 MEN2A patients in present study. Undiscovered oncogene products located on these chromosomal regions might be associated with activation of *menin* and *RET* for neoplastic transformation in MEN1 and MEN2A through signal pathways activated by ROS or replication stress.

Mutator genes were reported as control elements in *Zea mays* or male recombination mutation in *Drosophila*

melanogaster. These gene control elements, which are analogous to the DNA insertion sequences or transposable elements in bacteria, are capable of inducing chromosome rearrangements in mitotic cells. The formation of these mutator genes are of prime importance for explanation of hereditary tumors, which might depend on the cell type and stage of differentiation. Similar chromosome instabilities are reported in familial polyposis, familial retinoblastoma, Gardner's syndrome, Peutz-Jeghers syndrome and familial leukemias. The chromosome instability might predispose to neoplasias and permit and promote the clonal growth of selected cells. The relationship between chromosome instability and cancer incidence is remained to be resolved.

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