Enhancement of Sister Chromatid Exchanges in Peripheral Blood Lymphocytes of Thyroid Cancer Patients *in Vitro*

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Abstract: The aim of our study was to determine the chromosomal fragility of thyroid cancer patients *in vitro*. The cytogenetic methodology used in our study, was the SCEs (sister chromatid exchanges technique). FPG (fluorescence plus giemsa) method, was used in order to dye chromosomes. PRI (proliferation rate index), MI (mitotic index), AGT (average generation time) and PDT (population doubling time) were also counted. CPT-11 (Irinotecan), an original alkylotic agent, was used in our experiments as a positive control. Samples from 19 thyroid cancer patients and 13 healthy donors were controlled. The levels of SCEs were analyzed as a quantitative index of genotoxicity and the PRI and the MI were estimated as qualitative indices of cytostaticity and cytotoxicity, respectively. After CPT-11 addition in human lymphocytes cultures, the chromosomes of the cancer patients had a significant increase of the mean SCEs frequency. PRI and MI of treated with CPT-11 and untreated lymphocytes of thyroid cancer patients were significantly lower than those of healthy donors. The results suggested that peripheral lymphocyte chromosomes of cancer patients are highly fragile and the alkylotic agents increase their genetic instability. It appears that the increased SCE levels in peripheral blood lymphocyte chromosomes of thyroid cancer patients often result in chromosomal brittleness.

Key words: CPT-11, proliferative rate index, mitotic index, peripheral lymphocytes, genotoxicity, cytotoxicity, cytostaticity.

Abbreviations

BrdUrd	5-bromodeoxyuridine
FPG	Fluorescence Plus Giemsa
PRI	proliferation rate index
MI	mitotic index
SCE	sister chromatid exchange
CPT-11	irinotecan

1. Introduction

Thyroid cancer is the most common malignancy of endocrine organs worldwide, and it is considered as the most responsible cause of endocrine cancer related mortality [1]. Its incidence rate is steadily increasing over the past several decades according to data from the National Statistical Office and from the National Cancer Institute's SEER (Surveillance, Epidemiology

and End Results) database, since the incidences are now nearly 3 times of the early 1970s [2-4]. Furthermore, for unknown reasons, thyroid cancer is almost three times more prevalent in women than men. Thyroid cancer is one of the most common types of cancers among women according to the European Cancer Observatory and more specifically the EUCAN (incidence in men is 0.7 and in women is 3.1, among 100.000) [5].

Prognostic factors of thyroid cancer patients include tumor size, tumor histological type, extrathyroidal extension, distant metastasis, the presence of lymphnode metastasis and the existence of oncogenes [6]. Among these factors, the main cause of mortality in thyroid cancer is due to distant disease with about 50% survival at 3.5 years [7].

There are 4 basic types of thyroid cancer: the PTC (papillary thyroid carcinoma), which is the most

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common type of thyroid cancer and it's the one that we examined, the FTC (follicular thyroid carcinoma), the ATC (anaplastic thyroid carcinoma) and the MTC (medullary thyroid carcinoma).

Different types of treatment are available for patients with thyroid cancer. Surgery (lobectomy, lymphadenectomy, near-total thyroidectomy, total thyroidectomy) is the most common treatment of thyroid cancer. The radiation therapy has two types, the external (x-rays and γ -radiation) and the internal radiation (ingestion or inhalation of radioiodine) therapy and it uses high-energy x-rays or other types of radiation to kill cancer cells or keep them from growing. One of the most important treatments is the RAI (radioactive iodine) therapy, which is the most common therapy to follicular and papillary thyroid cancers. Other types of treatments are the thyroid hormone therapy, which removes hormones or blocks their action and stops cancer cells from growing, the chemotherapy, which uses drugs to stop the growth of cancer cells and the targeted therapy, which uses drugs or other substances to identify and attack specific cancer cells without harming normal cells [8, 9].

The risk of the thyroid cancer can be affected by many factors. A variety of external agents, including dietary (obesity is associated with thyroid cancer development in both women and men), environmental, and chemotherapeutic agents, can induce breakage at fragile sites. [10].

The present study investigated the chromosomal instability of thyroid cancer patients in comparison with healthy people.

SCEs (sister chromatid exchanges) reflect the exchange of genetic material between two identical sister chromatids at homologous loci, before they differentiate into two separate chromosomes during the metaphase stage of mitosis cell, which involves DNA breakage and reunion [11]. With the formation of SCEs, the cell tries to repair the unrepaired damage of DNA. So, an increased number of SCEs, means the increased number of damages in DNA, which may be caused by

various factors and simultaneously it indicated an increased incidence of double-strand breaks. The existence of sister chromatid exchange and their analysis is an excellent tool for the quantitative and the qualitative evaluation of the DNA damage caused by physical, chemical or biological mutagenic agents.

The sister chromatid exchange phenomenon is widely used as a simple, rapid, reliable and sensitive indicator of chromosome (DNA) instability, since the SCE patterns can reveal a general genome instability [12]. Variations in DNA repair mechanisms have been implicated as causing genetic susceptibility associated with cancer [13]. There were observations, which lead to the conclusion that substances that enhance the creation of chromatid exchanges, act as mutagens and carcinogens and they multiplied the main interest around creating an exchange [14, 15]. SCE in peripheral lymphocytes has been widely used to evaluate exposure to mutagens and carcinogens [16, 17]. It is a very sensitive and relatively quick process that can be used in tumor or normal cells, to in vitro and in vivo experiments [18].

The method used for the observation of chromatid exchanges, is the method based on differential staining of sister chromatid, after the impact of the Fluorescence Plus Giemsa technique. According to this method, it is possible to visualize the phenomenon of chromatid exchanges with the application of the fluorescent dye and the BrdUrd (5-bromodeoxyuridine), which then leads to the different coloring of the two sister chromatid (Figs. 1 and 2). This differentiation staining is observed because the culturing of the cells occurs in the presence of BrdUrd, which as an analog of the deoxythymidine and since it is in excess of the environment of the cells, it replaces the thymidine in the nascent DNA chain during the replication phase [19, 20].

There are many agents that cause damage to the chromosomes and these agents can be divided in two categories: the S-independent agents, which are independent from the S-phase of the cell cycle and the

Fig. 1 A second-division metaphase after Fluorescence Plus Giemsa staining of a healthy person blood lymphocyte, where all chromosomes have one lightly stained chromatid and one heavily stained chromatid (arrows show sister chromatid exchanges). Original magnification 1000X.

Fig. 2 A second-division metaphase after Fluorescence Plus Giemsa staining of a thyroid cancer patient blood lymphocyte, where all chromosomes have one lightly stained chromatid and one heavily stained chromatid (arrows show sister chromatid exchanges). Original magnification 1000X.

S-dependent agents, which are dependent from the synthetic phase of the cell cycle. The S-independent agents damage the DNA during the whole cycle, causing chromosome ruptures and other abnormalities. On the contrary, the S-dependent agents, as the age and the sex, or the UV light, in order to act into the cells, must pass through the synthetic phase [21]. Other agents like the alcohol, the smoking, the antineoplastic drugs and many diseases are also responsible for an increased frequency of SCE's [22, 23].

Many works have been published about the relation

between SCE's and cancer. The first work was conducted from Kurving in 1978, who showed that the mean frequency of SCE's in normal lymphocytes was 6,1/cell, while in patients with cancer it was 12,7/cell. Many researchers later, found a similar increase of the SCE's levels in different kinds of cancers, such as the thyroid cancer. Studies over the past several decades have shown a correlation between fragile sites and cancer-specific chromosomal aberrations [24, 25].

The aim of this study was to determine, whether genetic impairment and DNA damage have an effect on the pathogenesis of thyroid cancer. Antineoplastic drugs generally enhance the frequency of SCE, and CPT-11 (Camptothecin-11) is such a drug. It is an antineoplastic drug with anti-tumor applications. This drug is included to the inhibitors of the topoisomerases and more specifically of the topoisomerase I. It is a semisynthetic analogue of the camptothecin-11, which is an alkaloid isolated from the plant *Camptotheca acuminate* and into our body it is converted to the drastic metabolite SN-38, inhibiting the action of the topoisomerase I. CPT-11 has been extensively used in chromosomal fragility studies, in order to investigate if diseases and various physical, chemical and biological agents affect its mutagenic efficiency [25-27].

2. Materials and Methods

2.1 Clinical Data

This study was conducted between January 2013 and August 2014 in the Genetic Laboratory of the Medical Department, in Alexandroupolis and the blood samples from the patients that were used for this study were taken from the Anticancer Hospital of Pireaus "Metaxa", in Athens. We performed SCE analysis in 19 non-smoking (13 women and 6 men) patients with papillary thyroid cancer, with mean age 47 years old (20-65) and 13 non-smoking (10 women and 3 men) healthy non-smoking blood donors, used as controls, with mean age 33 years old (22-59). The patients were selected from non-smoking and nonalcoholic subjects.

None of the subjects had a history of viral infection, bacterial infection or any metabolic diseases. The patients had not been treated with chemotherapy, radiotherapy or thyroidectomy during the last 4 months. The patient and control groups were chosen for their similar habits. The Anticancer Hospital “Metaxa” approved the human study. All patients were analyzed prior to treatment.

2.2 In Vitro Sister Chromatid Exchange Analysis

For SCE analysis, we used heparinized peripheral venous blood. The blood cultures were prepared in accordance with the standard protocol on peripheral blood cultures and all the operation took place in the continuous flow stream chamber. For each blood sample there were 2 cultures, the controls and the CPT-11s.

Cultures of peripheral lymphocytes were prepared in universal containers by adding 11 drops of whole blood to 5 mL of Chromosome Medium L (RPMI-1640, penicillin and streptomycin, fetal bovine serum, phytohaematoglutinin-L, L-glutamine). There were incubated at 37 °C for 72 h. Cells were allowed to proliferate for at least 2 mitotic cycles, in the presence of BrdU, which had a final concentration of 5mg/mL. 50 ml of CPT-11 was added at the beginning of the culture period, to the 1 out of 2 cultures, from each sample. After 70 h, 0.5 µg/mL of colcemid was added for 2 h, and at the end of the incubation period cultures were harvested. Cultures were maintained in the dark to prevent or minimize photolysis of BrdU. Further processing was conducted, including hypotonic treatment, with 0.075 KCl solution, for 25 minutes, which spreads and hemolyzes the red blood cells, centrifugation, fixation for three times with methanol:acetic acid (3:1 v/v) slide preparation and a slightly changed FPG (fluorescence plus giemsa) staining technique for the detection of SCEs.

2.3 Statistical Analysis

Cells on the first, second, third and subsequent

mitotic divisions were counted. Three indices were evaluated: 1) SCEs, which is a qualitative and quantitative index of genotoxicity; 2) the PRI (proliferative rate index), which is a qualitative index of cytostaticity; 3) the MI (mitotic index), which is a qualitative index of cytotoxicity; 4) AGT (average generation time), which is studied as the ratio of BrdU time (h) and proliferate rate index; and 5) PDT (population doubling time), which is the time, in which the cells divide (i.e. in one cell cycle of 24 h) [28-31]. All indices were evaluated in each subject for either treatment. Mean SCE values were evaluated only in the suitable second division metaphases, because only in these metaphases were we able to observe and count SCEs. In order to establish PRI, 200 cells were counted and the following formula was used: $PRI = (M1 + 2M2 + 3M3_{+}) / N$, where M1 is the percentage of cells in the first division, M2 in the second and M3₊ in the third and subsequent divisions, while N is the total number of cells counted (i.e., (M1 + M2 + M3₊)). The MI for 2 000 activated lymphocytes was determined for all cultures.

In order to compare various treatments, a logarithmic transformation of the SCE values was performed using the one-way ANOVA (analysis of variance) and the Tukey's test for the pair-wise comparisons, of the SPSS 17.0 version for Windows. The level of significance from all tests was determined with *p* value. Less than 0.05 of the *p* value was considered as statistically significant.

The evaluation of MIs and PRIs was based on the χ^2 -test. A probability of *p* < 0.05 was considered statistically significant.

Finally, the AVG (average generation time) and the PDT, were counted, using the following types:

$$AGT (h) = 72 \text{ h (BrdU time) } / [PRI]$$

$$PDT (h) = 24 \text{ h } / [PRI] [28-31].$$

3. Results

SCEs frequency, PRI and MI values of lymphocyte cultures from thyroid cancer patients immediately after

the diagnosis and before any treatment are illustrated in Tables 1A and 2A, whereas the values of the same indices for lymphocyte metaphases derived from healthy donors and used as controls are illustrated in Tables 1B and 2B. Values in baseline of each column are the mean values for each in vitro treatment of chromosome preparations derived from the two groups examined, the thyroid cancer patients and the healthy donors. Each value of SCEs in Table 1 (A); (B) is the mean value of 20 metaphases in the second mitotic division and each MI value is the mean value of 2000 mesophasic nuclei. Also each PRI value in Table 2 (A); (B) is the mean value of 200 metaphases. AGT and PDT values of lymphocyte cultures from thyroid cancer patients and from the healthy donors, are also shown in Table 2 (A); (B).

As shown in Table 1B, the levels of SCEs in chromosomes of thyroid cancer patients are quite high with a mean value of 10.56 SCEs per metaphase. When

CPT-11 was added in lymphocyte cultures SCEs were increased significantly ($p < 0.01$) and reached a mean value of 31.68 SCEs per metaphase showing that the antineoplastic's genotoxic action affects chromosomes fragility significantly as shown by the increased values of SCEs index.

As shown in Table 1B the MI value for cultured lymphocytes of thyroid cancer patients is 35.42 and it was statistically significant ($p < 0.01$) decreased to 26.53 when CPT-11 was added.

The mean PRI value—which is shown in Table 2B—of lymphocytes derived from thyroid cancer patients is 2.38. After CPT-11 treatment the PRI value of lymphocyte cultures derived from thyroid cancer patients significantly ($p < 0.01$) decreased, comparing to that of control cultures, to 2.39.

Also from the Table 2 (A); (B) is obvious that both AGT and PDT are higher in the lymphocyte cultures from the thyroid cancer patients, in contrary to the

Table 1 Calculating the values of the two cytogenetic markers in vitro; SCEs/metaphase, and mitotic index (MI) on the human lymphocytes cultures from the healthy donors and the thyroid cancer patients.

A. Cytogenetic Indices from 13 Healthy Control Subjects				
	Mean SCE frequency/cell		Mitotic index (%)	
	SCEs $\pm$ SEM	Counted no. of metaphases per donor: 20	MI	Counted no. of interphase nuclei per donor: 2,000
Control	6.19 $\pm$ 0.21	Total counted: 260	39.88	Total counted: 26,000
CPT-11	28.88 $\pm$ 0.44	260	39.88	Total counted: 26,000
B. Cytogenetic Indices from 19 Patients with Thyroid Cancer				
	Mean SCE frequency/cell		Mitotic index (%)	
	SCEs $\pm$ SEM	Counted no. of metaphases per donor: 20	MI	Counted no. of interphase nuclei per donor: 2,000
Control	10.56 $\pm$ 0.21	Total counted: 380	35.42	Total counted: 38,000
CPT-11	31.68 $\pm$ 0.3	Total counted: 380	26.53	Total counted: 38,000

Table 2 Calculating the values of the three cytogenetic markers in vitro; PRI (Proliferation rate index), AVG (average generation time) and PDT (population doubling time), on the human lymphocytes cultures from the healthy donors and the thyroid cancer patients.

A. Cytogenetic Indices from 13 Healthy Control Subjects			
	Proliferation rate index		Average generation time
	PRI	Counted no. of metaphases per donor: 200	PDT
Control	2.44	Total counted: 2,600	29.50
CPT-11	2.35	Total counted: 2,600	30.63
B. Cytogenetic Indices from 19 Patients with Thyroid Cancer			
	Proliferation rate index		Average Generation Time
	PRI	Counted no. of metaphases per donor: 200	PDT
Control	2.38	Total counted: 2,600	30.25
CPT-11	2.39	Total counted: 2,600	30.12

lymphocyte cultures of the healthy donors. For the cultures of the healthy donors, as it occurs from the Table 2A the AGT is 29.50 and the PDT is 9.83, which is significantly lower ($p < 0.05$) from the lymphocyte cultures of the healthy donors in which was added CPT-11, where the AGT is 30.63 and the PDT is 10.21.

The SCEs, MIs, PRIs, AVGs and PDTs were counted for lymphocytes of healthy individuals and are shown in Tables 1A and 2A. The mean value of the SCEs frequency of lymphocytes of control cultures is 6.19 SCEs/cell, which is significantly ($p < 0.01$) lower than that of lymphocytes treated with CPT-11, which was calculated 28.88 SCEs/cell.

The mean MI value of lymphocytes obtained from healthy donors is 39.88, whereas the value of the same index for lymphocytes from the same donors and treated with CPT-11 is statistically significant ($p < 0.01$) with mean MI value at 33.19.

PRI mean value of lymphocytes of control cultures, as shown in the Table 2B, is 2.44 and statistically significant ($p < 0.05$) lower than of cultures where CPT-11 was added, where the PRI mean value is 2.35.

From the Table 2B, it is also resulted that the values of AGT and PDT of the lymphocyte cultures of the cancer patients are higher ($p < 0.05$) than those of the lymphocyte cultures of the thyroid cancer patients with CPT-11, since the AGT and PDT values of the thyroid cancer patients are 30.25 and 10.08 respectively, whereas in the CPT-11 cultures of the thyroid cancer patients, the value of the AGT is 30.12 and the value of the PDT is 10.04.

The chromosomes of thyroid cancer patients break more easily than those derived from healthy individuals. The mean SCEs frequency of thyroid cancer patients lymphocytes of control cultures, calculated 10.56 SCEs/cell (Table 2A), is significantly ($p < 0.01$) higher compared to the 6.19 SCEs/cell, of healthy individuals (Table 1A). The same happens between the SCEs frequencies of the treated with CPT-11 lymphocytes of the two groups (Table 2 (A); (B)). Lymphocytes of cancer patients and healthy

individuals have almost the same ability to promote their cell cycle as shown by their almost equal mean values of PRI which are 2.38 and 2.44 respectively. PRI value of lymphocytes of thyroid cancer patients treated with CPT-11 is 2.39 (Table 2B) and significantly ($p < 0.01$) lower than that of healthy individuals, which PRI is 2.35 (Table 2A). The MI value of thyroid cancer patients lymphocytes is 35.42 (Table 1B) and statistically significant ($p < 0.01$) of that of healthy donors, which is 39.88 (Table 1A). After the lymphocytes were treated with CPT-11 the MI values were decreased. The mean MI value of lymphocytes of thyroid cancer patients treated with CPT-11 is 26.53 and statistically significant ($p < 0.01$) lower than those of healthy individuals treated with CPT-11, which mean MI is 33.19. The AGT value of thyroid cancer patients lymphocytes is 30.25 and statistically significant ($p < 0.01$) of that of healthy donors, which is 29.50. Finally the PDT value of the thyroid cancer patients lymphocytes is 10.08 and statistically significant ($p < 0.01$) in contrast to the PDT of the lymphocyte cultures of the healthy donors, which is 9.83.

4. Discussion

Thyroid cancer is the most significant endocrine cancer and it is the most responsible cause of endocrine cancer related mortality [1], with a continuous increasing rate in Europe [2-4]. It is found that thyroid cancer is much more common in women [5]. It has been associated with nutrition, environmental and chemotherapeutic agents [10]. Cancer results from genetic or epigenetic alterations in a wide range of genes that control cell division, cell differentiation and cell death.

The significance of SCEs is obvious in the fact that their evaluation could solve problems that concern chromosome structure and function, as well as DNA transcription. Their main use is to study the harmful action of several physical or chemical agents on DNA. Furthermore, it has been proved that there is a linear

relationship between SCEs and mutations.

An increase in the levels of chromatid exchanges in metaphase chromosomes directly reflects DNA damage, which indicates the failure of pre-replicative repair mechanisms to achieve full recovery of the damaged site, thus leading to double-strand breaks [32]. In order for eukaryotic cells to overcome the potentially mutagenic and cytotoxic properties of double strand breaks, caused by DNA-damaging agents, various repair pathways are employed along with homologous recombination. In this particular pathway, sister chromatids play a crucial role [33, 34]. This is because cohesion proteins hold together sister chromatids during the G2 phase, and something that enables recombination events to take place. On the other hand, the fact that another DNA repair pathway, or mismatch repair pathway, preventing recombination between homologous sequences might actually be the reason why recombination between homologous chromosomes is prevented [35].

It has been found that SCEs frequency is increased in cancer patients lymphocytes suffering from many kinds of cancers such as breast cancer, uterine cervix, ovarian, prostate and thyroid cancer [36, 37].

The antineoplastic agent, CPT-11, has been demonstrated as causing cytostatic effects in human peripheral lymphocytes either to healthy donors or to patients [38]. Furthermore, cytotoxicity is simultaneous with CPT-11. Irinotecan increases SCEs frequency in a wide range of cells and is widely used in order to measure chromosome fragility. The use of irinotecan as a positive control is widespread in a plurality of tumor and normal cell lines [39]. In this way, we are given the option of evaluating the chromosomal fragility in patients with thyroid cancer preventive, before the start of any treatment.

This study provides an evaluation of SCEs frequencies, PRIs, MIs, AGTs and PDTs in peripheral blood lymphocytes and their genome fragility of thyroid cancer patients just after the diagnosis and prior every medication, surgery or other kind of therapy.

Baseline SCEs, MI, PRI, AGT and PDT were significantly higher in patients than in healthy donors used as controls, who were carefully selected (not smokers, without any infection for at least a month and not taking medication). Moreover, thyroid cancer patients show higher levels of SCEs in their lymphocytes [36].

The patients with thyroid cancer illustrated increased instability. They were more inclined to have induced DNA damage, in contrary to the healthy people when cells were treated with CPT-11 (see Table 1A). Feedback control operates at the mitotic entry checkpoint to prevent cells with damaged DNA from entering mitosis until the damage is repaired. CPT-11 is a cross-linking clastogen. Induced by CPT-11, cross-links lead to SCE formation through a cross-link bypass model, rather than homologous recombination, which has been proposed as one of the mechanisms of SCE formation [40].

The levels of SCEs of patients lymphocytes are significantly higher than those of the control group. The high SCEs frequencies show that cancer patients lymphocytes chromosomes are very fragile and even more than those of healthy individuals.

PRIs and MIs of cancer patients lymphocytes are significantly lower than that of healthy donors, with and without the CPT-11 treatment, resulting to their inability to promote their cell cycle as fast as the healthy lymphocytes do.

Cell division tracking provides a unique opportunity for the analysis of cell growth kinetics. These include the average time between successive divisions (AGT) and time required to increase the total cell number to two-fold (PDT) [30]. AGT revealed significant differences in a dose dependent manner in the CPT treated cultures. As far as the population doubling time, it shows how much the carcinogen is affecting the growth rate of cells, including the cell cycle kinetics. This analysis provides specific and quantitative information about cell cycle arrest induced by an agent that can be used to assess the contribution of cell cycle

arrest effect to the overall growth inhibition. AGTs and PDTs of cancer patients lymphocytes are significantly higher than those of healthy donors, pointing out that the thyroid cancer affects the time between the divisions and the growth rate of cells.

In conclusion, in the present study SCEs were found significantly increased results in patients with thyroid cancer relatively to healthy controls which may be associated with the pathogenesis of thyroid cancer. Our findings illustrated a strong association between thyroid cancer patients and chromosomal instability. The patients were more inclined to have induced DNA damage due to their extraordinary higher increased SCE levels in peripheral blood lymphocytes. This condition could be the result of different spontaneously arising damages, which happen both to patient and healthy individuals. The present data established that SCEs are sensitive markers of chemotherapy related damage, especially in the case of alkylating agents like CPT-11 and are indicative of the attention that doctors must pay in order to avoid killing healthy cells of the patient such as lymphocytes. SCEs can be considered as an additional diagnostic tool for identifying genetic instability by a combination of spontaneous or induced genotoxic, cytotoxic and/or cytostatic effects on patients with thyroid cancer. Finally, we propose that the identified instability must be strongly considered before subscribing any medication. Further investigation in other antineoplastic drugs acting in another way but alkylation on cells and in vivo clinical studies are needed to validate our results.

5. Conclusion

In conclusion, we suggested that peripheral lymphocyte chromosomes of cancer patients are highly fragile, which is expressed by enhanced genotoxicity, cytostaticity and cytotoxicity of lymphocytes and the alkylating agents increase their genetic instability. It appears that the increased SCE levels in peripheral blood lymphocyte chromosomes of thyroid cancer patients often result in chromosomal brittleness.

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