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Human NY-ESO-1 gene encodes for an antigen belonging to cancer/testis (CT) protein. NY-ESO-1 gene is expressed in many human cancers with various histological types, but not in normal tissues except testis. The NY-ESO-1 antigen is a potential target for cancer immunotherapy. We investigated the expression of gene NY-ESO-1 in the samples of hepatocellular carcinoma (HCC) tissues and adjacent liver tissues by RT-PCR. 56 of 124 tumor tissues from HCC patients were NY-ESO-1 positive (45.2%). In contrast, NY-ESO-1 mRNA was not detected in any of the adjacent tissues. A cDNA fragment of 329bp was amplified by RT-PCR from each of the 56 positive samples and sequenced. Four samples were found to have point mutations at 2 sites resulting in a substitution of one amino acid residue. Such substitution is predicted to have a profound effect on the protein structure and hence on the functional motif in the protein. To distinguish whether the substitution is a point mutation or a polymorphism present in normal population, the genomic DNA fragment has been amplified from 10 samples of the peripheral blood mononuclear cells (PBMCs) of healthy donors and sequenced. All the tested samples displayed the same sequence as that had been reported. Thus, the base pair change occurred in HCCs is maybe a point mutation. In HCCs, the expression of NY-ESO-1 has no correlation with other parameters tested, such as tumor markers (aFP, AFU), metastasis, recurrence, and tumor diameter ($P<0.05$). The relatively high rate of NY-ESO-1 gene expression in HCCs indicates that NY-ESO-1 protein may be a vaccine candidate to be developed as immunotherapy for HCC patients.

Key words: NY-ESO-1, RT-PCR, hepatocellular carcinoma, gene expression, immunotherapy.

Introduction

A number of human tumor antigens which are recognized by CD4/CD8 T cells or autologous antibodies have been increasingly identified by various approaches for the last decade [1]. Of the more than ten genes or gene families coding for CT antigens, NY-ESO-1 was identified from esophageal cancer by a serological approach (SEREX), which is based on the screening of recombinant tumor cDNA expression libraries for specified interactions with autologous serum antibodies [2]. A large-scale serologic survey of over 700 tumor patients' sera showed that anti-NY-ESO-1 antibody(Ab) was often present in patients with NY-ESO-1$^+$ tumors, with 30% to 50% of those patients in the study having detectable NY-ESO-1 Ab [3]. In comparison, Ab responses to other CT antigens were found to be much lower. More importantly, the NY-ESO-1 protein contains both B cell epitope and T cell epitope capable of simultaneously inducing both humoral and cellular immune response in vivo and in vitro [4; 5]. It is further confirmed by the identification of a 10-mer antigenic peptide in NY-ESO-1 protein capable of presenting HLA-A31 molecule to induce specific CTL response [6]. Furthermore, recent report has demonstrated that fucoidin enhances the cross-presentation of NY-ESO-1 to T cells, resulting in an increase of T-cell cytotoxicity against NY-ESO-1 expressing human cancer cells [7]. NY-ESO-1 has been reported to be frequently expressed in melanoma [2; 8], breast cancer [9; 10], neuroblastoma [11], esophageal squamous cell carcinoma [2], prostate cancer [12; 13], transitional cell carcinoma [14; 15], and lung cancer [16]. There have been so far several reports describing a moderate rate of NY-ESO-1 expression in HCC samples [17–20]. We extended this study to investigate NY-ESO-1 cDNA expression in 124 human HCC tumor tissues and their respective adjacent liver tissues.

Materials and Methods

Patients

All 124 HCC patients aged 24 to 70 years old were admitted for surgery at Xiangya Hospital of Central South University from May 2005 to January 2009. The clinicopathological data, including gender, stage of disease, tumor invasion, TNM classification, etc. are listed in table 1. Normal controls of PBMCs were collected from healthy donors at the age of 20 to 24 years old to detect NY-ESO-1 genomic DNA.

Cell Lines

The melanoma cell line SK-MEL-37, which expresses NY-ESO-1 mRNA, were kindly provided by Xiangya Hospital of Central South University for Cancer Research.

Tissue Samples

Samples of HCCs and liver tissues were collected from pa-

Table 1

The Relationship between the Expression of NY-ESO-1 Gene and the Clinicopathological Parameters

Clinicopathological parameters	Expression of NY-ESO-1 gene				
	Positive	Negative	Positive rate, %	χ^2	p
Total	56	68	45.2	—	—
Sex					
male	46	53	46.5	0.5617	0.45
female	10	15	40.0%		
Age					
< 40 yr	17	21	44.7	0.9497	0.33
> 40 yr	39	47	45.3		
Tumor diameter					
< 5 cm	10	15	40.0	0.5617	0.45
> 5 cm	46	53	46.5		
Tumor embolism of portal vein					
yes	7	10	41.2	0.7223	0.40
no	49	58	45.8		
Tumor capsule					
integrity	25	42	37.3	0.0569	0.81
incompletion	31	26	54.4		
Tumor number					
single	43	47	47.8	0.3408	0.56
multiple	13	21	38.2		
HBV infection					
yes	49	61	44.5	0.6993	0.40
no	7	7	50.0		
HCV infection					
yes	9	5	64.3	0.1268	0.72
no	47	63	42.7		
Cirrhosis					
none/light	27	32	45.8	0.8980	0.34
middle/heavy	29	36	44.6		
AFP					
normal (< 20 ng/l)	16	25	39.0	0.3345	0.56
high	40	43	48.2		
AFP mRNA					
positive	46	54	46.0	0.7017	0.40
negative	10	14	41.7		
AFU					
normal (<198 nKat)	5	14	26.3	0.0728	0.79
high	51	54	48.6		
Survival time					
< 2 years	34	37	47.9	0.4802	0.49
> 2 years	22	31	41.5		
Metastasis and recurrence					
< 1 year	23	16	59.0	0.0363	0.85
> 1 year	33	52	38.8		

tients who were undergoing routine hepatic surgery at Xiangya Hospital (n=204). Among them, 124 patients suffered form HCC, 37 were cirrhosis without HCC, and the remaining 43 were non-cirrhotic liver diseases. HCC tissues and the adjacent tissues (5 cm away from the tumor margin) were collected from patients undergoing HCC resection. Written informed consent was obtained from each patient. The resected tissues were stored in liquid nitrogen after snap frozen until being used for RNA extraction.

Isolation of RNA

Total RNA was extracted from liver tissue samples, using RNeasy mini kit and QIAshredder (QIAGEN). Tissue samples were homogenized in liquid nitrogen and lysed in TRIZOL (1 ml / 50-100 mg tissue, GIBCOL BRL). Total RNA was isolated by phenol/chloroform extraction, precipitated with isopropanol, washed with 75% EtOH, and finally dissolved in RNase-free dH_2O. RNA concentration was assessed using UV spectrophotometry at OD_{260}. The ratio of $OD_{260/280}$ was 1.78-1.82. The RNA samples were analyzed in 2% agarose gel electrophoresis to determine the RNA integrity.

Synthesis of cDNA

RNA samples (5 ug) were mixed with oligo-dT15 (0.5 ug/ul, Promega, USA) and random hexamers (0.5 ug/ul, Promega, USA), and added with RNase-free dH_20 to 12 ml. The mixture was heated to 70°C for 10 minutes and quickly chilled on ice. The contents in the tube were collected by brief centrifugation, and then added with the following reagents: 4 ml 5× reverse transcriptase buffer (250 mM Tris-HCL [pH 8.3 at room temperature], 375 mM KCL, 15 mM $MgCL_2$), 2 ml 0.1 M DTT and 1ml 10 mM dNTP Mix (10 mM for each dATP, dGTP, dCTP and dTTP at neutral pH). The contents were mixed gently and in-

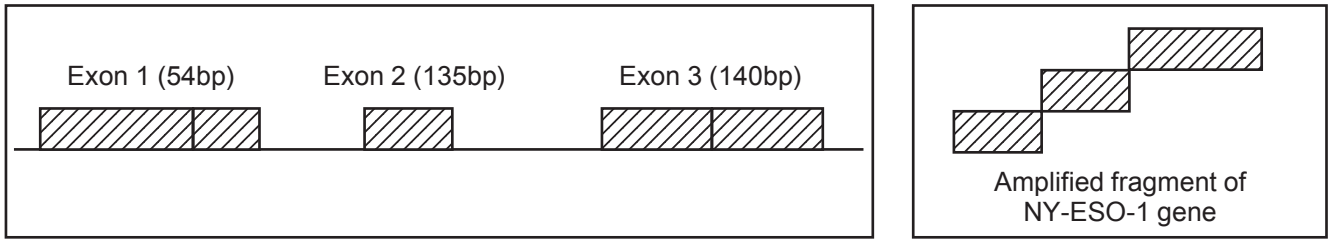

Fig. 1. The structure of NY-ESO-1gene (329bp).
Note. There are three exons in NY-ESO-1 gene; underlined frame indicates the amplified fragments

cubated at 42°C for 2 minutes, and then added with 200 U of superscript II reverse transcriptase (GIBCO BRL, USA), 20 U RNase inhibitor (RNAsin, Promega), and RNase-free dH_2O to a total volume of 20 ml. cDNA synthesis was performed at 42°C for 50 minutes. The RT enzyme was heat-inactivated at 70°C for 15 minutes.

PCR Amplification of G3PDH cDNA

To assess the integrity of the cDNA, amplification reactions (50 ml) were performed with the following reagents: 1 ml cDNA, 10 mM glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers (sense: 5'- ACC ACA GTC CAT GCC ATC AC-3'; antisense: 5'- TCC ACC ACC CTG TTG CTG TA-3'), PCR buffer (20 mM Tris-HCL [pH 8.4], 50 mM KCl), dNTP (1 ul, 10 mM, Promega) and 2.5 U Taq polymerase (GIBCO BRL, USA), and by Thermal Cycler (Perkin-Elmer 9700, USA) under the following conditions: 94°C for 20 seconds, 65°C for 40 seconds, and 72 °C for 40 seconds (35 cycles, followed by a final extension at 37°C for 7 minutes.

PCR Amplification of NY-ESO-1 cDNA

The amplification reactions (50 ml) contained 1ml cDNA, 10 mM NY-ESO-1 primers (sense: 5'-ATG GAT GCT GCA GAT GCG G-3'; antisense: 5'- GGC TTA GCG CCT CTG CCC TG-3' (Fig. 1), 5 ml 10 × PCR, 3 ml 25 mM $MgCl_2$, 1 ml 10 mM dNTP Mix (10 mM for each dATP, dGTP, dCTP and dTTP at neutral pH), 2.5 ml DMSO, 1.5 U Tag DNA polymerase(GIBCO BRL, USA) and PCR H_2O. The samples were denaturation at 94°C for 2 minutes, and the amplifications were performed under the following conditions: 94°C for 40 seconds, 60°C for 40 seconds, and 72°C for 30 seconds (35 cycles), followed by a final extension at 72 °C for 7 minutes. The amplified cDNAs were run in 1.5% agarose gel with a NY-ESO-1 band of 329 bp.

Southern Blot Analysis

The RT-PCR products were fractionated by electrophoresis in 2% agarose gels and transferred to nitrocellulose membranes at 4°C for 16 hours. A NY-ESO-1 cDNA fragment (25 ng) of 329 bp, derived from RT-PCR products and confirmed by DNA sequencing, was labeled with [^{32}P]dCTP (HIGH PRIME kit, Boehringer Mannheim GmbH, Germany) and used as probe. The cDNAs immobilized on the membranes were prehybridized with 6 × SSC, 2 × Denhardts, 0.1% SDS, and 100 mg/ml denatured fragmented salmon sperm DNA at 68°C for 16 hours, and then hybridized with the probe at 68°C for 8 hours. After hybridization, the membranes were sequentially washed twice at 68°C in 2 × SSC and 0.1% SDS, 30 minutes each, and twice at 68°C in 0.1 × SSC and 0.1% SDS, 1 hour each. The membranes were exposed to X-ray film at -70°C with intensified screens to obtain an autoradiogram.

Cloning and Sequencing of NY-ESO-1 cDNA

The purified NY-ESO-1 cDNAs obtained from PCR amplification were cloned into a pGEM-T vector (Promega) by T4 DNA ligase and amplified in E. coli, DH5a. The positive colonies were selected with EcoR1 digestion of miniprep DNA. The putative NY-ESO-1 cDNA samples were sequenced with T7 and M13 sequencing primers using an automatic gene sequencer (Visible Genetics Inc., Canada).

Analysis of NY-ESO-1 Gene Sequence in Genomic DNA

To determine polymorphism or point mutation of NY-ESO-1 variants, human genomic DNA was extracted from PBMCs obtained from 10 healthy donors using Trizol Reagent (GIBCO BRL). The human genomic DNA samples were boiled for 10 minutes and purified through Sephadex G-50 column. The primers (sense, 5'-ACC TCG CCA TGC CTT TCG -3'; antisense, 5'-GTC GGA TAG TCA GTA TGT TGC C-3') were designed according to the sequence of the second exon harbouring the mutated site (Fig. 2). The amplifica-

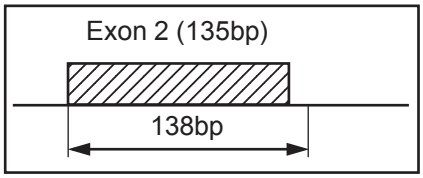

Fig. 2. The amplified fragment of Exon 2
Note. The length of Exon 2 is 135bp; the arrows indicate the amplified fragment.

tion reactions (50 ml) contained 5 ml 10 × PCR buffer, 3 ml 25 mM $MgCl_2$, 1 ml 10 mM dNTP Mix (10 mM for each dATP, dGTP, dCTP and dTTP at neutral pH), 1.5 U Tag DNA polymerase, 0.5 ml of each primer, 35 ml PCR water and 1 ml genomic DNA template. The amplified DNAs were run in 2.0% agarose gel with a positive band of 138bp (Fig. 3). The positive PCR products were ligated to a pGEM-T Easy vector and sequenced as described above. The putative NY-ESO-1 genomic cDNA samples were sequenced with T7 and M13 sequencing primers using an automatic gene sequencer (Visible Genetics Inc., Canada).

aFP mRNA detection

The total RNAs was extracted from PBMCs and reverse transcribed into cDNAs. The aFP mRNA was detected by amplification using nested RT-RCR.

Results

Expression of NY-ESO-1 in HCC

After the amplification by RT-PCR with the designed primers, a 329 bp product that the expected size of NY-ESO-1 cDNA was amplified from the NY-ESO-1 positive cell lines SK-MEL-37. Of the 124 HCC samples, 56 (45.2%) were found to express NY-ESO-1 mRNA, shown as a single band of 329 bp in the RT-PCR product, and each gave a strong and specific signal of the same size in Southern blot hybridization. In contrast, none of the adjacent liver tissues was NY-ESO-1 positive by RT-PCR (Fig. 4), nor in Southern blot hybridization (Fig. 5). Furthermore, all the 80 samples of liver tissues from non-cirrhotic (n=43) and cirrhotic patients (n=37) were NY-ESO-1 negative by RT-PCR.

NY-ESO-1 variants in HCC

All of the 56 NY-ESO-1-positive PCR products were cloned into the pGEM-T vector and transfected into DH5a. Three clones from each individual PCR product were picked up for DNA sequencing. The data were compared with GenBank database. The sequences of 156 clones from 52 samples were identical to the second exon of NY-ESO-1 cDNA. However, the sequences of 9 clones from 3 samples (H2, H28, H29) were shown to have point mutations at 2 sites (A347T, A432G, Fig. 6); the A347T mutation did not affect the coding amino acids, whereas the A432G mutation resulted in a substitution of threonine to alanine at position 127. Another sample (H10) exhibited only one point mutation as A432G. Notably, the A432G mutation in NY-ESO-1 cDNA was identified in each of the four samples, resulting in an identical substitution of amino acids. As it was independently confirmed by experiments, in which 3 clones were picked up and sequenced for each sample, this point mutation was unlikely to be caused by PCR artifacts.

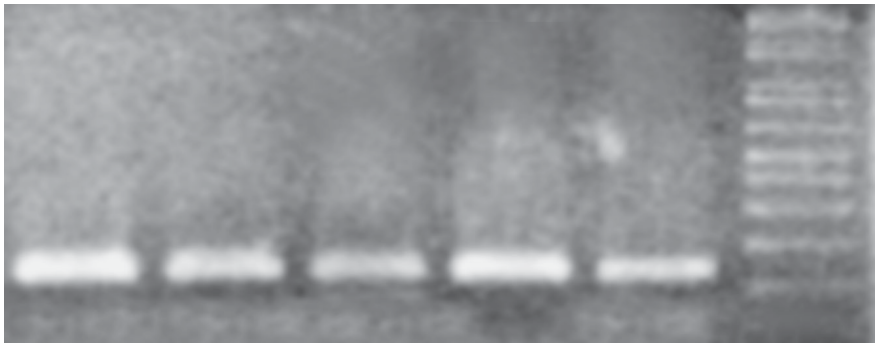

Fig. 3. The PCR results of genomic DNA

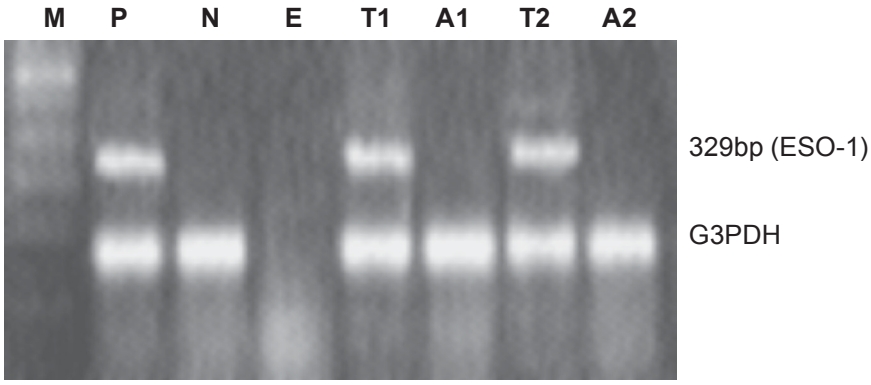

Fig. 4. The RT-PCR results of NY-ESO-1

Note. M, marker; P, positive control; N, negative control; E, empty control; T, tumor tissue; A, adjacent non-tumor tissue.

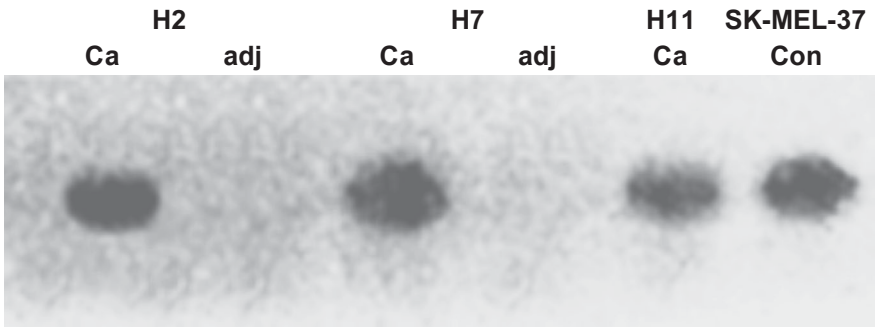

Fig. 5. The Southern Blot results of NY-ESO-1

The Relationship between NY-ESO-1 Expression and Clinicopathological Parameters

The expression of NY-ESO-1 mRNA was not correlated with other clinicopathological parameters, such as the tumor marker aFP tested at both protein and mRNA levels, AFU, metastasis, recurrence, tumor diameter, etc. (see Table 1).

Discussion

To accurately estimate the frequency of NY-ESO-1 expression in the HCCs of Chinese patients, the possibility that the RNA samples were contaminated with genomic DNA was excluded by control assay, in which no target band was detected in the PCR products amplified from RNA samples without prior reverse transcription. Our results showed that NY-ESO-1 was expressed in 45.2% (56/124) of the Chinese HCCs. In contrast, NY-ESO-1 mRNA was detected in none of the 124 adjacent tissues samples. To ensure the results' reliability, all positive PCR products were subjected to DNA sequencing and confirmed to be NY-ESO-1 cDNA fragments.

A total of 168 clones from 56 samples of the NY-ESO-1 positive RT-PCR products have been sequenced (3 clones were picked up from each positive RT-PCR product). The sequences of 156 clones from 52 samples were identical to the corresponding region of NY-ESO-1 cDNA in Genbank database. However, 3 samples (H2, H28, H29) were shown to have point mutations at 2 sites (A347T, A432G) in exon 2. The first point mutation of A347T did not lead to a change of amino acid residue, whereas the second point mutation (A432G) resulted in a substitution of one amino acid residue (Thr127Ala). In addition, one sample (H10) was shown to have only one point mutation of A432G, resulting in the substitution of threonine to alanine. To exclude any errors caused by PCR amplification, three clones from each individual positive PCR product of the four samples

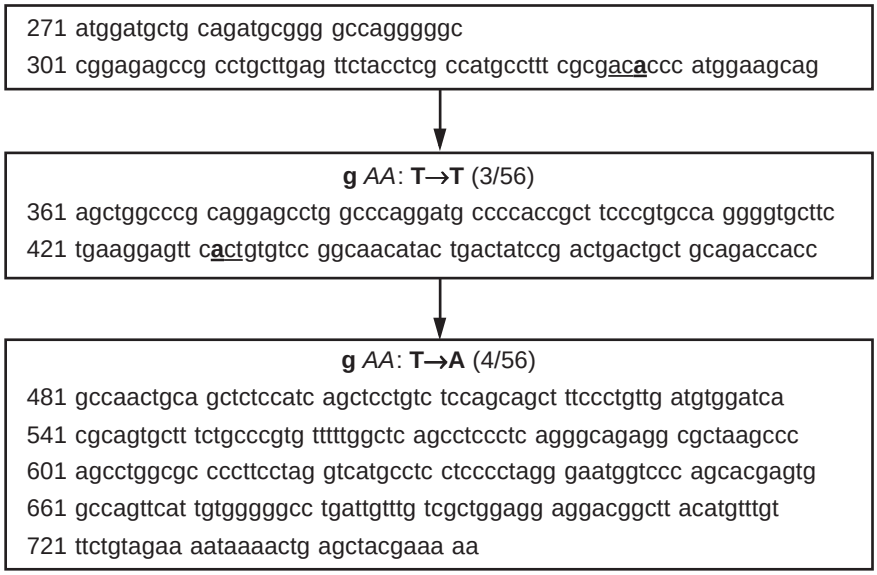

Fig. 6. The mutation positions of NY-ESO-1 gene
Note. A was replaced by T at position 347 (3/14), with no change of amino acids; A was replaced by G at position 432 (4/14), and the amino acid changed from Threonine to Alanine. Both of the two sites simultaneously mutated in three samples.

were randomly picked up for DNA sequencing, and all gave the same results. Besides, Taq plus DNA polymerase was used in PCR to minimize mismatches of base pairs during the amplification cycles. Thus, the base pair changes are unlikely to be PCR artifacts. More studies are needed to determine whether this cDNA sequence pattern reflects a gene polymorphism or should be attributed to point mutation occurred in HCC.

The substitution of threonine to alanine at position 127 may have implications in the biological properties of the protein. Based on the analysis of the linear and 2-demensional structure by Gene Runner (Fig. 7), it shows no change in protein hydrophilia;

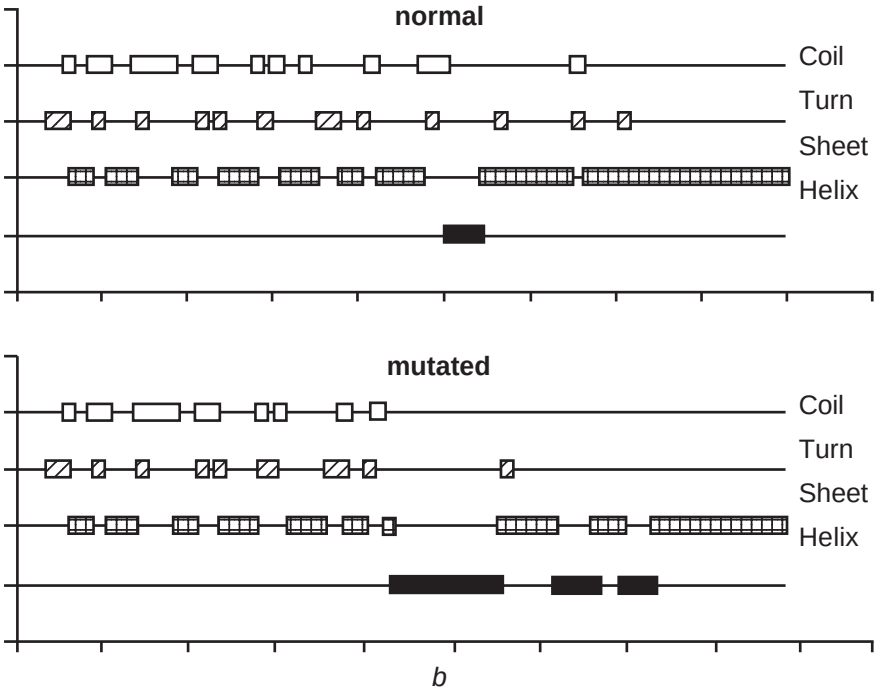

Fig. 7. The analyses of the linear and 2-demensional structures of the normal and mutated NY-ESO-1 genes by Gene Runner

however, the α-helix and β-sheet in the secondary structure of the variant NY-ESO-1 protein is predicted to be changed. These changes in the secondary structure may lead to an alternation in the tertiary structure, and hence, affect the exposure of antigenic epitopes or form new ones.

Currently, the clinical HCC diagnosis is based on disease history, liverish background, AFP, and CT scan. It is reported that NY-ESO-1 specific antibodies were detected in the serum of a part of patients whose NY-ESO-1 expression was positive, while the antibodies were not detected in the patients whose NY-ESO-1 expression were negative. Though the biological function of NY-ESO-1 protein has not been fully understood yet, the expression of NY-ESO-1 mRNA in liver tissues is indicative of hepatoma. Detection of NY-ESO-1 expression, therefore, adds a new parameter for the diagnosis of HCC. It is reported that HLA-A2-restricted T cells from HCC patients target NY-ESO-1, but exist in an exhausted state that might require additional activation to restore function [21], and currently, there are several ongoing cancer vaccine trials based on NY-ESO-1 that are showing promising results [22–24]. NY-ESO-1 may be a good candidate for vaccine development in the immunotherapy for HCC patients.

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