SAGE-Hindawi Access to Research
Stem Cells International
Volume 2010, Article ID 532704, 8 pages
doi:10.4061/2010/532704

Research Article

Noncultured Autologous Adipose-Derived Stem Cells Therapy for Chronic Radiation Injury

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Received 7 June 2010; Revised 4 August 2010; Accepted 28 September 2010

Academic Editor: Kotaro Yoshimura

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Increasing concern on chronic radiation injuries should be treated properly for life-saving improvement of wound management and quality of life. Recently, regenerative surgical modalities should be attempted with the use of noncultured autologous adipose-derived stem cells (ADSCs) with temporal artificial dermis impregnated and sprayed with local angiogenic factor such as basic fibroblast growth factor, and secondary reconstruction can be a candidate for demarcation and saving the donor morbidity. Autologous adipose-derived stem cells, together with angiogenic and mitogenic factor of basic fibroblast growth factor and an artificial dermis, were applied over the excised irradiated skin defect and tested for Patients who were uneventfully healed with minimal donor-site morbidity, which lasts more than 1.5 years.

1. Introduction

There is an increasing worry on radiation injuries probably caused by nuclear power plant (NPP) reactor accidents, therapeutic irradiation for malignancy, and interventional radiology (IRV) of unexpectedly prolonged fluoroscopic procedures for cardiovascular diseases such as arrhythmia, ischemic heart diseases, or nuclear medicine of overdose intake of the radioactive for nuclear medicine of internal radiation therapy. The problems are concerning chronic radiation injury as well as how to heal such local and systemic injures acutely. Local chronic radiation injury is resistant to conventional therapeutic modalities such as flap coverage or skin grafting because the deteriorated margins are sometimes indistinguishable from normal intact tissue, and thus sufficient enough debridements are not obtained with surgeons' naked eyes.

These conditions should be treated properly for the sake of life saving and improvement of local wound healing [1]. However, data of total evidence-based clinical analysis were not established yet. Authors' institute, Nagasaki University, is selected as a global strategic center for radiation health risk control by the Japan's Ministry of Education, Culture, Sports and Technology from FY 2007 to 2011 and exploring to establish such therapeutic regimens, to prevent the radiation injuries, and possibly to regenerate medical and surgical therapy for radiation injuries by using patients' own adipose tissue-derived stem cell therapy.

Often seen chronic radiation injuries are well handled by sufficient enough blood supply to the radiated tissues, especially in the cartilage, bare bone, and hardened scar tissues. For this purpose, local, distant, and microsurgical vascularized flaps are applied. Recent development of microvasculature of the skin and soft tissues including the connective tissues plays major roles in attributing to accelerate local wound healing. Also, externally administered angiogenic growth factor such as basic fibroblast growth factor (bFGF) together with temporal wound coverage of artificial skin substitute is very effective for those patients with severe injuries, patients with comorbidities, who are

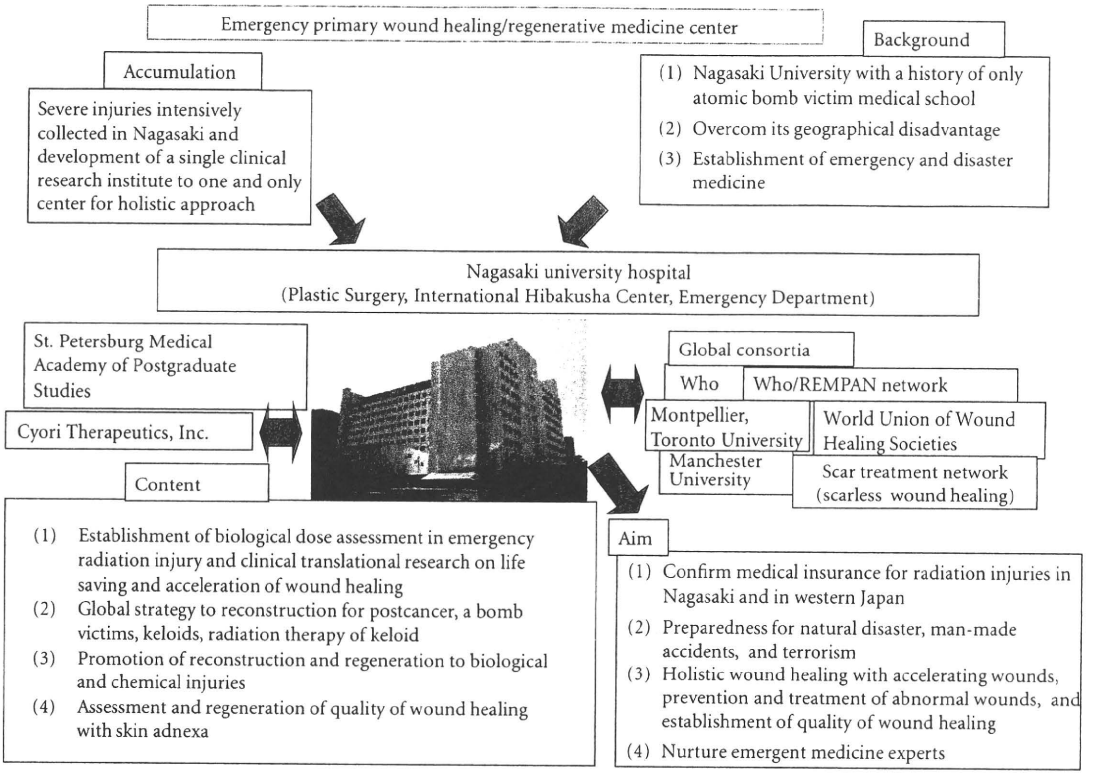

FIGURE 1: Strategy of emergency radiation injury. Collaborative work with highly established international centers and organ is proposed.

intolerant to the extensive and long surgeries [2]. Here, chronic radiation-injured wounds are tested with non-cultured autologous adipose-derived stem cells and clinical implications are discussed.

2. Materials and Methods

2.1. Treatment of Chronic Local Radiation Injury with Conventional Methods and Stem Cells. Often experienced in radiation therapy for malignancy, cardiovascular modalities should be categorized as difficult wounding with poor vasculature or less healing potentials.

From January 1990 to April 2007, 10 (8 females and 2 male) patients who demonstrated chronic radiation injuries such as telangiectasia, xerosis, epidermal atrophy karatoses, and fibrosis as well as deep ulcers in the costal ribs and sternum by adjuvant radiation therapy after mastectomy and prolonged fluoroscopic procedures for cardiovascular diseases were surgically treated.

Other selective clinical cases used angiogenic growth factor namely human recombinant basic fibroblast growth factor (rh-bFGF), which is clinically approved and widely used for clinical wounds in Japan with skin substitutes, which are also clinically available not only in Japan but also in many other nations including USA, the majority of EU nations, and several Asian counties, and the effectiveness of using the artificial skin substitutes in the chronic radiation injuries is temporal coverage and sustainability of both internal and external cells and growth factors. Therefore, combined use of bFGF and artificial skin substitute leads to improved quality of wounds (scar tissue) as well as facilitated wound healing [3].

One case was treated with non-cultured autologous adipose-derived stem cell (ADSC) for chronic sacro-coccygeal radiation ulcer in 2008, which was caused by a therapeutic radiation at fractionate 50 Gy at 40 years previously.

2.2. Methods. This study was approved by the Ethics Committee of the Nagasaki University Hospital, and written informed consent was obtained from all patients (approved no. 08070296) and partly supported by the Global COE (Center of Excellence) Program E08, Global Strategic Center for Radiation Health Risk Control, and it was funded by the Japan Society for the Promotion of Science. This national research grant enables us to investigate 3 main themes related to radiation health risk: (1) atomic-bomb disease followup cohort research with over 60-year continuous research history, (2) radiation basic science, and (3) international radiation health research. Especially, this radiation regeneration research was involved in further international collaboration framework under international organizations such as WHO (World Health Organization) and IAEA (International Atomic Energy Agency) (Figure 1).

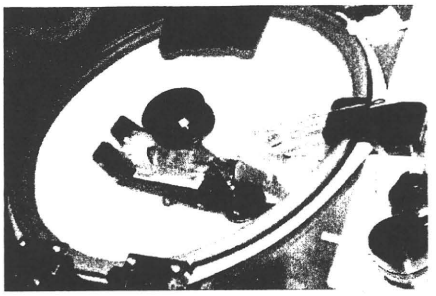

FIGURE 2: The Adipose-derived stem cells are processed in a closed-circuit machine within 1.5 hours.

2.3. Harvesting of Adipose Tissue by Liposuction and Isolation of ADSCs. 3–5 mm incisions, two incisions in the abdomen, four incisions in the thigh, and two incisions in the gluteal region, were made on the abdominal region, the thigh, and the gluteal region. The subcutaneous layer was infiltrated with a lactated Ringer's solution with addition of 0.5 mL of epinephrine and 25 mL of 1% lidocaine per 500 mL. Adipose tissue was suctioned using an 18-G Becker cannula with a 50 mL syringe. Total 250 gram-fat tissues, 120 grams from the abdominal region, 80 grams from the gluteal regions, and 50 grams from the thighs were harvested.

ADSCs were isolated from the suctioned adipose tissue by using the Celution system. (Cytori Therapeutics, Inc., USA). Briefly, the suctioned adipose tissue was introduced into the Celution cell-processing device, which automatically and aseptically extracts and concentrates the mononuclear fraction of adipose tissue and removes unwanted or deleterious cells, cell and matrix fragments such as lipids. By using the Celution system, a 5 mL solution is added to isolated ADSCs in about one and a half hour (Figure 2). The whole procedure is in a closed circuit and this reduces the chance of the contamination.

The small portion of processed ADSCs was used for the ex vivo cell culture and confirmed the proliferation and differentiation potential. The ADSCs-rich fraction was then plated onto collagen type-I-coated plastic culture flasks in a serum-free medium for primate embryonic stem cells (Primate ES medium, RiproCELL, Tokyo), and the cells, clonally expanded, were collected and stored in Liquid Nitrogen as the primary ADSCs. ADSCs were subcultured when they reached to 80% confluence. Cells were treated with trypsin/EDTA solution, neutralized with tripsin-neutralizing solution, and collected by centrifugation for 5 minutes at 1,200 rpm. The pellects were resuspended in a fresh medium; the number of cells was counted, and 3×10^5 cells were plated into T25 flasks (25 cm^2) for subculture while the rest of the cells were stored in liquid nitrogen.

2.4. Adipose-Derived Stem Cell Grafting and Postoperative Management. For the scaffold purpose, we used the artificial dermis (Terudermis, Olympus-Terumo Biomaterials Co., Ltd., Japan) (Figure 3). The Terudermis is composed of two layers: a lower layer of bovine atelocollagen and an upper layer comprising a silicone sheet which protects against infection and dryness from the outside. After minimum debridement, the Terdermis was multilayered and stacked over freshly debrided wounds. The silicone sheets were removed except top Terudermis. The two-thirds of isolated ADSCs alone were injected; around the debrided wounds, at the base of the wounds, and into Terudermis. Another one-third of ADSCs was mixed with the autologous adipose which was rinsed with a lactated Ringer's solution. In the Celution system, after isolating ADSCs, the disposable cell collection plastic case one was again used to mix the suctioned fat, which is rinsed separately in the 50-cc syringe and repeated until the oil droplets are removed. After being mixed, it was injected into a zone of hard fibrotic tissue around the debrided wounds in 2-cm width in all directions.

2.5. Angiogenic Growth Factor and Basic Fibroblast Growth Factor (bFGF). Genetically recombinant human bFGF (Fiblast, Trafermin) was purchased from Kaken Pharmaceutical Co., Inc (Tokyo, Japan). The Freeze-dried bFGF was dissolved in 5 mL of benzalkonium chloride containing solution right before the first use and stored at 4°C for one day, with 300 μL sprayed over 30 cm^2 area from 5 cm distance, and 0.3 mL per day of this solution was applied over the wound. One week after removing the silicone layer, human recombinant fibroblast growth factor (bFGF: Fiblast, Kaken Co., Ltd., Japan) (Figure 4) was sprayed. The wound was covered with nonadherent occlusive foam dressing.

3. Results

3.1. Treatment of Chronic Local Radiation Injury with Conventional Method. All wounds were healed after several surgical modalities. None of the cases was healed with single procedure (2 to 6 surgeries, mean 4.3).

Of our cases, one breast-cancer patient was treated by a standardized Halsted method with major and minor pectoralis muscle, radical neck, and axillary and internal mammary lymph node dissections. This patient has undergone 50-Gy fractionate radiation therapy postoperatively. The radiated area showed chest fistula deep to the pleura with surrounding unhealthy hardened scar tissue and chronic inflammation.

The whole affected area was sequentially excised in 3 reconstructive surgeries, starting with rectus abdominis musculocutaneous flap, then latissimus dorsi musculocutanous flap, and finally with groin-free flap. In the course after each surgery, the margin of the flap was partially dehiscent and necrotized, which required further touchups? The total number of the reconstructive surgery was 6 (Figure 5).

3.2. Treatment of Chronic Local Radiation Injury with Adipose-Derived Stem Cells. Regeneration method with patient's own non-cultured ADSCs was planned for a patient underwent 50-Gy fractionate radiation therapy for uterine cancer 40 years ago. The pigmented sacrococcygeal region appeared with central intractable wound. Necrotized bone and fascia muscle along with malodour were observed. The ADSCs-treated chronic radiation wounds underwent

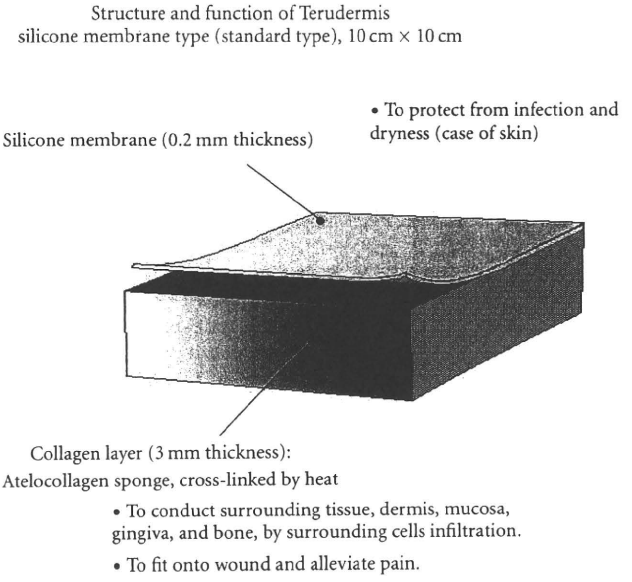

FIGURE 3: Freeze-dried bilayer artificial dermis made of bovine dermis. The outer membrane of silicone layer is easily removed and easily soaked with cell-containing solution.

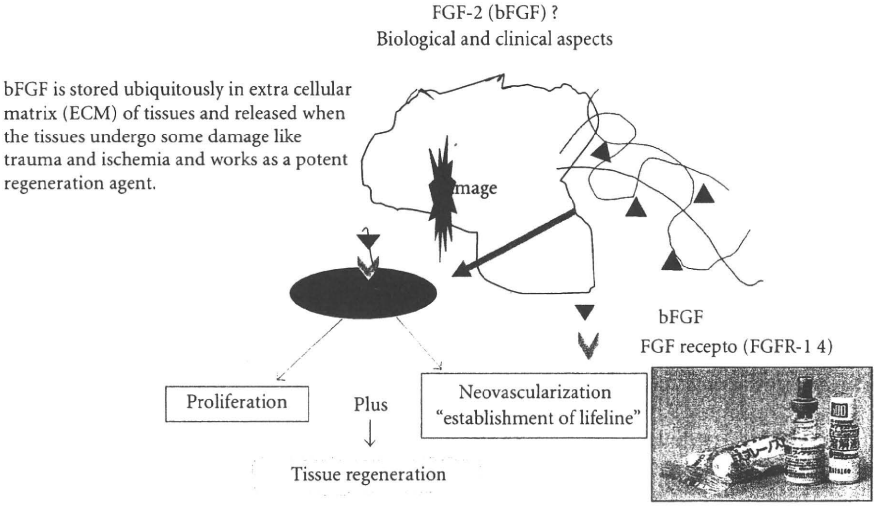

FIGURE 4: Commercially available growth factor and basic fibroblast growth factor (bFGF). Mode of action is explained and the mechanism is proposed.

debridement to remove unhealthy superficial necrotized bone, fascia, periosteum, and muscle. 3.8×10^7 cells in 5-mL of final volume from 250 mL of subcutaneous aspirated fat obtained from nonradiated area were used. Some ADSCs were directly injected in wound bed and margins; others were soaked with the artificial dermis. In a few days postoperatively, the silicone upper layer of the artificial dermis (Terdermis) was removed, and bFGF was sprayed over the regenerated wound for three weeks. There was no significant adverse effect neither in donor site or treated wound. The wound was healed uneventfully by day 82 and no sign of recurrence appeared, but the regenerated tissue developed mature in 1.5 years (Figure 6).

4. Discussion

Local radiation injuries caused during medical therapy for malignant tumors [4] and heart disease [5] may be accompanied with systemic symptoms of hematologic, neurologic, and gastrointestinal symptoms such as neutropenia, thrombopenia, fatigability, nausea, and diarrhea by contact to the

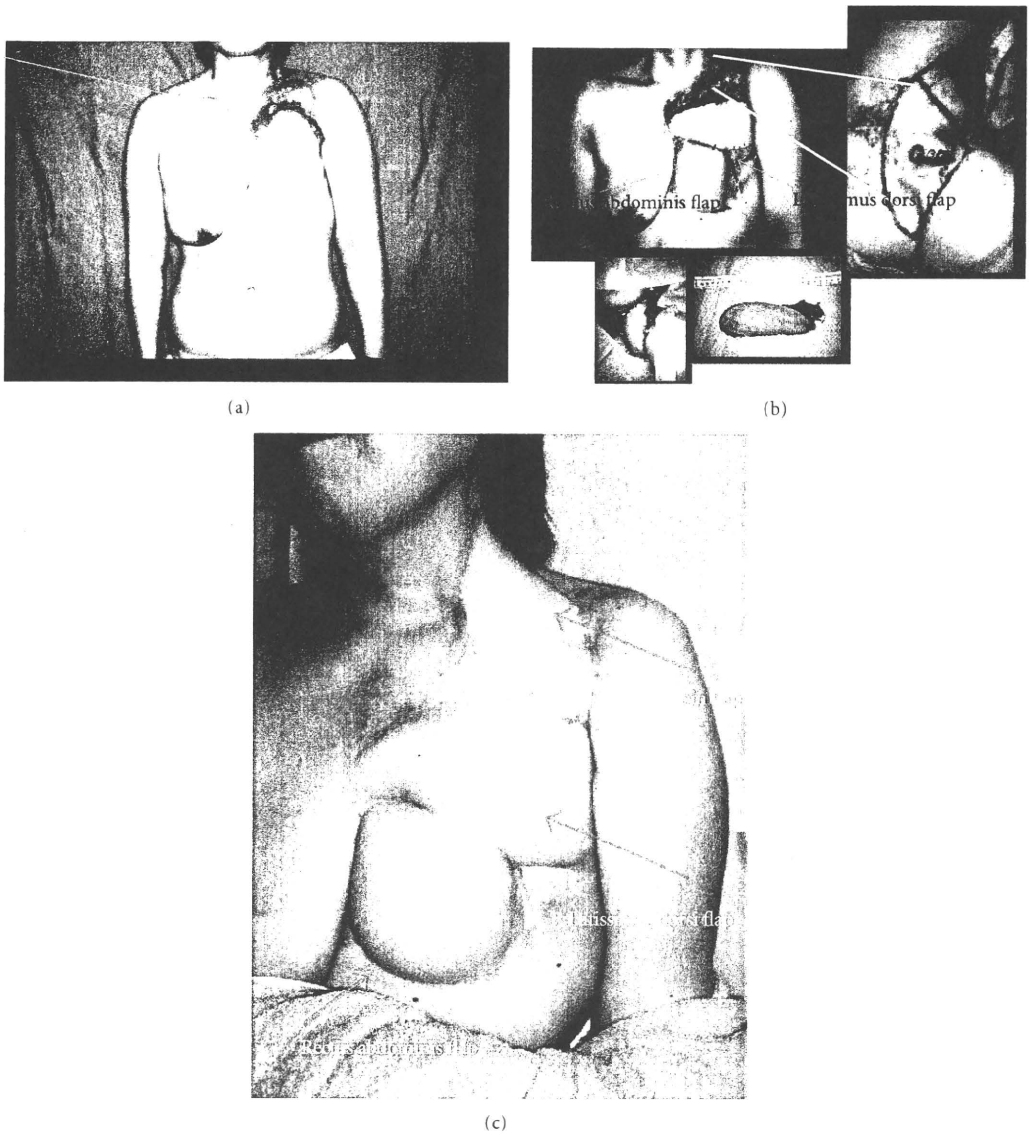

FIGURE 5: 55-year-old woman underwent a left breast cancer surgery by a standardized Halsted methods, followed by 50-Gy fractionate radiation therapy 15 years previously. (a) The chest demonstrates fistula to the costal rib and adjacent to the pleura as the arrow depicts, and the surrounding tissues were firm and various-degree inflammation existed. (b) Sequential three major flaps (rectus abdominis, latissimus dorsi, and free groin flap) are used for total coverage. (c) In 7 years postoperative view. There is irregularity of the scar margins.

scrap yard radioactive wastes without notice [6] or exposure to the radiation accidents [7] by touching gammagraphy radioactive source by mistake [8]. Since locally radiated tissues show decreased or insufficient vascularity and tissue damage, demonstrating erythema, teleangiectasia, pigmentation, or dermal atrophy, once wound is developed, it is often intractable and further leading to tissue necrosis, infection, and later fibrosis in demonstrating chronic radiation injury syndrome [9]. Therefore, radiation-injured wounds tend to persist for a long time, show impaired healing, and be prone to recurrence even by minor trauma. Radiated wounds are treated by adequate debridement both in the depth and in the width and covered with well-vascularized tissues or by cultured bone-derived mesenchymal stem cells [8]; however, the long-term outcome is not warranted, and donor-site morbidity and the duration for treatment are sometimes concerned, especially for the aged patients or patients who somehow have problems in harvesting the donors or being limited due to the coexisting diseases. As seen in our reconstructive cases, the surgical modalities constantly required multiple surgeries partly due to the definitive damage-free margins of the affected tissue.

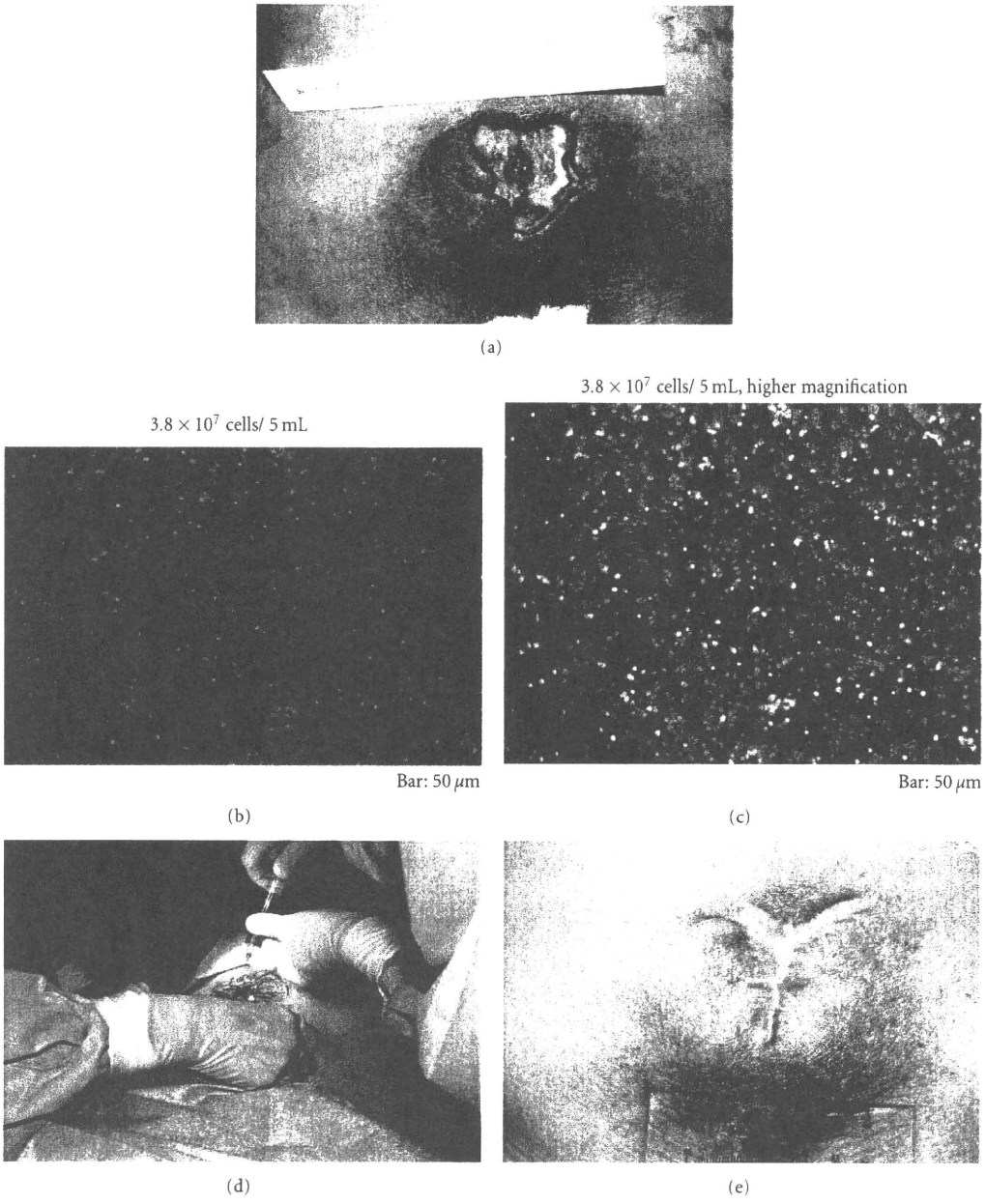

FIGURE 6: 89-year-old woman underwent a uterine cancer surgery followed by 50-Gy fractionate radiation therapy 40 years previously. (a) In 10 × 10 cm area of radiation, 5 × 10 cm area was exposed. Bone, fascia, and muscle as well as skin and fat were affected. (b, c, d) After careful debridement, 3.8×10^7 cells/5 mL were applied over the wound bed and margins and soaked with artificial dermis. In a few days postoperatively, bFGF was sprayed over the peeled-off inner regenerated tissue for 21 days. (e) In 1.5 years postoperative view. The regenerated tissue remained durable, soft, and pliable.

Application of Stem cell therapies for repair and regeneration has recently been investigated at a clinical level in variously defected or injured tissues, among which stem cells and adipose-derived stem cells (ADSCs) can be harvested with a minimally invasive procedure by liposuction procedure through a small incision. Similar to our method but in detail very different, Clinically purified autologous lipoaspirates were used as treatment for radiotherapy tissue damage of consecutive 20 patients. Indirectly, induced ADRCs have potential in cell therapy for radiation injury due to increasing neovascularization and retention of the fat property [10].

This enables us to adopt this regeneration method for patients with severe comorbidity such as elderly systemic disease and physical wasting state (data not shown). The

ADSCs contain several types of stem and regenerative cells, including endothelial and smooth muscle cells and their progenitors and preadipocytes [11]. The ADSCs have the capacity to differentiate into multiple lineages and cell types including mesodermal tissues such as fat, bone, cartilage, endothelial cells of endodermal origin, and neurons and epidermis of ectodermal origin as seen in the mesenchymal stem cells [12].

Management of radiation injuries composes two major parts. One is localized injuries and the other is of systemic injuries. Among localized radiation injuries, chronic injuries are more common in the medical field after caner radiation therapy. Usually management of these chronic wounds is well handled by well-vascularized tissue transfers as various plastic surgical procedures have proved. In consideration of each patient general condition and preference, the choice of therapeutic selections should be performed. On the other hand, when the local radiation injuries are encountered in an acute phase, there are high chances for innovative procedures using autologous stem cells. The hMSCs are resistant to radiation. We have previously demonstrated *in vitro* cell proliferation curve and are also able to produce protein avoiding cell apoptosis [13]. And the application of cultured bone-derived mesenchymal stem cells successfully healed severe local radiation wounds. However, the cultured stem cell therapy takes longer period as long as 16 days before cell therapy and required multiple (5 times) cell injections as well as 2 skin grafting, 2 flaps, and 1 artificial dermis coverage [14]. Also, increasing evidences demonstrate that ADSCs are similar to hMSCs in cell properties and characteristics both *in vitro* and *in vivo* [11]. ADSCs are highly yielding and less invasive for donor sites. The acute myocardial infarction porcine models by improving left ventricular function, perfusion, and remodeling [15]. When localized radiation was distant enough from the donor sites adipose tissues, immediate debridement and regeneration happens using adipose-derived stem cells, which are available for processing within 1.5 hours simultaneously in the same operation theater without cell culture since adipose tissues (fat tissues) are abundant in adult humans compared to other stem cell sources. In the limited clinical circumstances of high-risk patients such as elderly and chronic local infection, there is still opportunity of harvesting and processing the patient's own fat-derived stem cells successfully as seen in our case. Practically for emergency radiation injury cases, more abundant cell sources such as fat are the primary candidate for this purpose. The cell property and characterization of ADSCs are discussed and discussed either fresh or cultured [16]. The results from the clinical trial for acute myocardial infarction are expected and may be applicable for acute radiation injury treatment.

For treatment of systemic radiation injuries, stockpiled stem cells should be globally available through medical assistance network system under WHO-REMPAN, in which Nagasaki University is highly involved in its activity, or other international frameworks. Early resurfacing of the damaged skin and subcutaneous tissues is as important as hematological and intestinal system resuscitation [17].

Also, therapeutic guidelines for systemic radiation injuries are anticipated from practical and regulatory view points. Highlighting innovative technology and devices as well as currently existing medicines and devices is expected for the sake of preparing to treat "systemic" radiation injuries most effectively.

Therapeutic regimens of radiation injuries used to be dependent on each subspecialty in the medical filed such as internal medicine, radiology, and surgery.

Recent establishment of wound care specialty was mostly led by plastic surgeons, but other supporting specialists such as nurses, dermatologists, and gastrointestinal physicians and surgeons may be practically handling these rare but of significant impact "radiation injuries" as a interdisciplinary approaches. Therefore, more specialization for "radiation injuries" may be required.

Acknowledgment

Nagasaki University Global COE Program, "Global Strategic Center for Radiation Health Risk Control," by Japan Society for the Promotion of Science.

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Down-regulation of microRNA 10a expression in esophageal squamous cell carcinoma cells

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Received February 8, 2010; Accepted 26 March, 2010

DOI: 10.3892/ol_00000093

Abstract. This study identified significantly down-regulated microRNAs (miRs) specific for esophageal squamous cell carcinoma (ESCC) cells. Total RNA was extracted from ESCC cell lines (OE21 and TE10) and a non-malignant human esophageal squamous cell line (Het1A), and subjected to microarray analysis. Expression levels of miRs that showed significant down-regulation in ESCC cells compared to Het1A cells based on the comprehensive analysis were analyzed by quantitative reverse transcription polymerase chain reaction. Among the significantly down-regulated miRs, miR-10a expression levels in the five ESCC cell lines examined were significantly lower than in Het1A and the esophageal adenocarcinoma cells. Since miR-10a is a specific miR in ESCC, its clinical relevance was examined. Using ESCC tumor samples and non-cancerous tissue obtained endoscopically, the involvement of miR-10a in the clinicopathological findings was examined. MiR-10a expression was comparably down-regulated in the tumors of high-grade intraepithelial neoplasm and non-invasive ESCC, while the expression levels were elevated in the invasive ESCC tumors. Treatment with a demethylating agent, 5-aza-2'-deoxycytidine, restored miR-10a expression in OE21 cells. Only a modest additive or synergistic effect was observed in the presence of a histone deacetylase inhibitor, trichostatin A. These results imply that miR-10a may be differentially expressed in ESCC cells and may be involved in ESCC development and progression. The unique epigenetic regulation of miR-10a expression can be mediated via hypermethylation of the CpG islands proximal to its gene locus, at least in certain ESCC cells.

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Key words: microRNA, microRNA 10a, esophageal squamous cell carcinoma, DNA methylation

Introduction

Esophageal cancer is the eighth most common cancer and the sixth most common cause of cancer deaths worldwide (1). Although Barrett's adenocarcinoma is the most rapidly increasing cancer in Western countries (2), esophageal squamous cell carcinoma (ESCC) is still dominant in East Asia, including Japan (3). ESCC is often diagnosed at a late stage; thus, the prognosis of affected patients is unsatisfactory, despite the development of therapeutic options such as surgery, chemotherapy and radiotherapy (4). Consequently, there is a need for biomarkers to allow for a tailored, multimodality approach with increased efficacy. However, efforts to identify molecular markers in association with the pathogenesis of ESCC have been unsuccessful thus far (5).

MicroRNAs (miRs) are small, non-coding RNAs that negatively regulate gene expression via translational repression or messenger RNA degradation. More than 700 miRs have been identified and registered in humans, with each individual miR predicted to target multiple genes based on the seed sequence matches in their 3'-untranslated regions (UTRs) (6). MiRs are involved in biological and pathological processes, including cell differentiation, proliferation, apoptosis and metabolism (7), and they are emerging as highly tissue-specific biomarkers with potential clinical applicability for defining cancer type and origin (8,9). Accumulating evidence indicates that the deregulation of miRs is associated with human malignancies, suggesting a causal role of miRs in tumor initiation and progression (10) since they are able to function as oncogenes or tumor suppressors (11).

Pioneering studies on chronic lymphocytic leukemia (CLL) showed that miRs play a role in cancer pathogenesis and that the expression of miR-15a and miR-16-1 was deleted

in the majority of CLL case analyses (12). Further functional analysis identified an anti-apoptotic Bcl-2 as one of the actual targets regulated by these miRs, implying that miR-15a and miR-16-1 were tumor suppressor genes that deregulate cellular survival (12). Human *let-7* genes that map to regions are deleted in many cancer types, and the let-7 family may also function as tumor suppressors (13). A possible mechanistic explanation for this was provided by the discovery that RAS oncogenes are the targets of let-7 members (14). Similarly, the genomic locus encoding miR-34a is frequently lost in certain malignancies (13), and non-small cell lung tumors exhibit a reduced expression of miR-34b and miR-34c (15). miR-34 members were shown to be direct transcriptional targets of p53, a representative tumor suppressor protein (13,16) in that their ectopic expression induces p53 itself and its downstream targets and reduces p53-dependent apoptosis. These data establish the integration of certain down-regulated miRs into the tumor suppressive pathways (11,13).

Currently, there is limited information on the relationship between the pathogenesis of ESCC and miRs. Therefore, the present study was designed to identify the miRs that are specifically down-regulated in ESCC cells, possibly exerting regulatory activities.

Materials and methods

Cell lines and cultures. Human ESCC cell lines OE21, TE5, TE8, TE10 and TE11; 1 non-malignant human esophageal squamous cell line immortalized by SV40 infection (Het1A); 2 human Barrett's adenocarcinoma cell lines (Bic-1 and Seg-1); 3 human gastric adenocarcinoma cell lines (AGS, AZ521 and KATOIII); 2 colorectal adenocarcinoma cell lines (Caco-2 and DLD1); 1 human cervix epithelioid carcinoma cell line (HeLa); 1 human lung adenocarcinoma cell line (A549) and human hematological malignant cell lines (acute promyelotic leukemia, HL60; human T cell lymphoblast-like cell line, Jurkat and histiocytic lymphoma, U937) were cultured. The AZ521, KATOIII, DLD-1, HeLa, A549, HL60, and U937 cells were purchased from the Japanese Collection of Research Bioresources Foundation (Sennan, Japan). The OE21, Het-1A, AGS and Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The TE5, TE8, TE10 and TE11 cells were purchased from Riken Bioresource Center Cell Bank (Tsukuba, Japan). Bic-1 and Seg-1 were kindly provided by Dr D.G. Beer (Ann Arbor, MI, USA). The OE-21, TE5, TE8, TE10, TE11, Het-1A, U937, HL-60, DLD-1, Jurkat and KATOIII cells were grown in RPMI 1640 medium, while the HeLa, A549, and Caco-2 cells were maintained in Eagle's minimal essential medium. Both media were supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% glutamine, and all cell lines were cultured in a humidified incubator under 5% CO_2 at 37˚C.

Patients and clinical samples. Consecutively, 20 patients with ESCC or high-grade intraepithelial neoplasm (HGIN), or controls without the tumor who underwent esophagoscopy between June 2007 and May 2009, were recruited. After obtaining their informed consent, three biopsy samples were removed from the ESCC tumors and from normal-appearing esophageal mucosa under endoscopic observation. Of these samples, two were placed immediately into 1 ml of RNA (Applied Biosystems, Foster City, CA, USA) for RNA isolation at a later time point. The other sample was fixed in 10% formalin and embedded in paraffin for histopathology. The paraffin-embedded biopsy samples were cut into 5-μm sections and stained with hematoxylin and eosin.

RNA extraction. Total RNA, including miR from the tissue samples and cultured cells, was extracted using a mirVana RNA Isolation kit (Applied Biosystems) according to the supplier's instructions. The quality of the total RNA was determined on a Bioanalyzer (Bioanalyzer RNA Nano kit, Agilent, Santa Clara, CA, USA), and the RNA was quantified using a Nanodrop-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The extracted RNA samples were stored at -80˚C until use.

MicroRNA array hybridization and analysis. To find specific miRs for ESCC cells, total RNA was extracted from OE21 and TE10 cells, representative well- and moderately-differentiated human ESCC cell lines, respectively, and the non-malignant human esophageal squamous cell line, Het1A. The isolated RNA samples were subjected to a comprehensive analysis of miRNA expression patterns with microarray-based technology, an Agilent Human miRNA array chip version 1 (Agilent), containing 15,000 probes corresponding to 470 unique human miRs and 64 human viral miRs catalogued in the Sanger database version 9.1. An aliquot of 100 ng each of total RNA was treated with calf intestine phosphatase (GE Healthcare, Chalfont St. Giles, UK), denatured using DMSO (Sigma, St. Louis, MO, USA), and directly labeled with Cy3 using T4 RNA ligase (GE Healthcare). Labeled samples were hybridized to the miR array 8X15k (G4470A) platforms in SureHyb chambers (Agilent), washed with the buffer supplied (Agilent), according to the manufacturer's instructions, and scanned using an Agilent Scanner (G2505B). Data were extracted using Feature Extraction software 9.3 and GeneSpring software (Agilent). To identify miRs that were differentially expressed between the ESCC cell lines and Het1A cells, a supervised analysis was performed using significance analysis of microarrays (SAM, Stanford University, Stanford, CA, USA). The differences in miR expression were considered significant if the fold-change of expression values was >2.0 and the p value was <0.05 using the t-test.

Quantitative reverse transcription-polymerase chain reaction analysis for microRNAs. Expression levels of miRs that showed significant differences based on the microarray results were analyzed by quantitative reverse transcription-polymerase chain reaction (RT-PCR) using various human malignant cell lines, including ESCC, and non-malignant Het1A. cDNA was prepared from total RNA using a Taq Man MicroRNA Reverse Transcription kit (Applied Biosystems). Predesigned Taq Man MicroRNA assays including the primer set and Taq Man probe were purchased from Applied Biosystems. The reverse transcription reactions were performed in aliquots containing 50 ng total RNA, 1.5 μl 1X RT primer, 1 μl 10X RT buffer, 0.15 μl 100 mM dNTP, 1 μl reverse transcriptase and nuclease-free water added up to 15 μl at 16˚C for 30 min, followed by 42˚C for 30 min and 85˚C for 5 min. PCR reactions

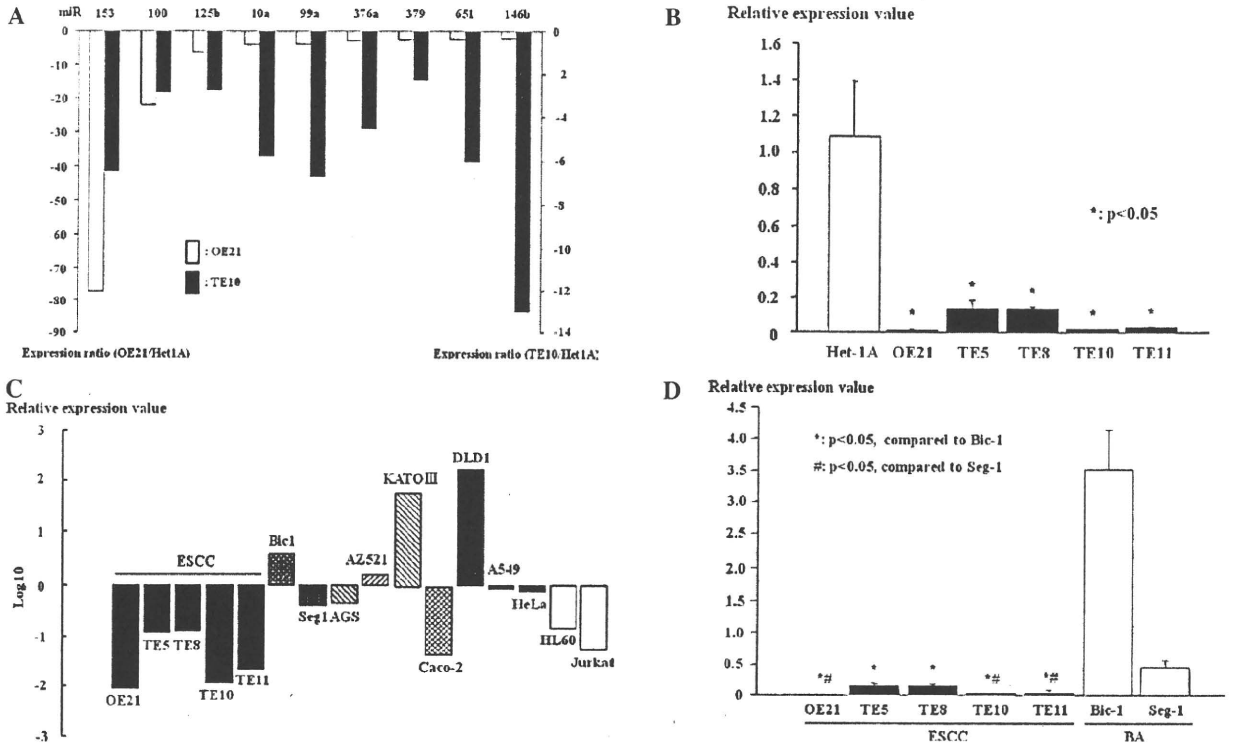

Figure 1. Based on microRNA (miR) microarray analysis, the expression of miR-153, -100, -125b, -10a, -99a, -376a, -379, -651, and -146b is significantly reduced in the two esophageal squamous cell carcinoma (ESCC) cell lines (OE21 and TE10) compared to Het1A cells (A). Quantitative reverse transcription (RT)-PCR shows a substantial decrease in the relative miR-10a expression levels in all ESCC cell lines (OE21, TE5, TE8, TE10 and TE11) compared to Het1A (B) and the Barrett's adenocarcinoma cells (C and D). The miR-10a expression levels did not necessarily decrease in the ESCC cells compared to those in the remaining malignant cell types examined (C).

were performed in 20-μl aliquots containing 1.33 μl of miR RT products with 18.67 μl of PCR master mixture (10 μl 2X Universal PCR master mix; 1 μl each primer; 1 μl Taq Man probe; and 6.67 μl nuclease-free water), and run in triplicate on the 7500 Real-Time PCR system (Applied Biosystems). Thermal cycling was initiated with a first denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The cycle passing threshold (Ct) was recorded for each candidate miR, and a small RNA, U6B, was used as the endogenous control for data normalization. Relative expression was calculated using the formula $2^{-DCt} = 2^{-(Ct, U6B - Ct,Specific)}$ as described in the ABI PRISM 7700 SDS relative quantification of gene expression protocol by PE Applied Biosystems. Similarly, total RNAs extracted from the neoplastic and non-neoplastic samples (esophagoscopic biopsies) were subjected to real-time quantitative RT-PCR for quantitation of miR-10a expression levels.

5-Aza-2'-deoxycytidine and trichostatin A (TSA) treatment. OE21 cells were incubated with or without 5-aza-2'-deoxycytidine (DAC) (1 or 5 μmol/l) for 96 h, followed by treatment with 0.5 μmol/l trichostatin A or vehicle for an additional 24 h. Total RNA was then extracted and subjected to the quantitative RT-PCR for measurement of the cellular miR-10a expression levels.

Statistical analysis. The differences between groups were analyzed using the unpaired, one-tailed, Student's t-test. Data are expressed as means ± standard error. Differences were considered statistically significant at $p<0.05$. All examinations were conducted according to Good Clinical Practice and the Declaration of Helsinki, and were approved by the Nagasaki University ethics committees.

Results

Specific down-regulation of microRNA-10a in esophageal squamous cell carcinoma. Based on the miR microarray analysis, the expression of miR-153, -100, -125b, -10a, -99a, -376a, -379, -651 and -146b was significantly (>2-fold) down-regulated in the ESCC cell lines compared to the non-malignant Het1A cells (Fig. 1A). On the other hand, miR-203, -429, -205, -200c and -141 were significantly (>2-fold) overexpressed in the two ESCC cell lines compared to non-malignant Het1A cells. We focused on the significantly down-regulated miRs, considering their possible regulatory actions for carcinogenesis (11,13). Real-time RT-PCR was used to quantify expression levels of miRs that showed significant reductions in expression on the microarray analysis. Among the corresponding miRs, only the miR-10a expression levels substantially decreased, respectively, in all of the ESCC cell lines (OE21, TE5, TE8, TE10 and TE11) compared to Het1A cells on quantitative RT-PCR (Fig. 1B). However, the miR-10a expression levels did not necessarily decrease only in the ESCC cells, and levels varied among the diverse malignant cell types examined (Fig. 1C). MiR-10a expression was

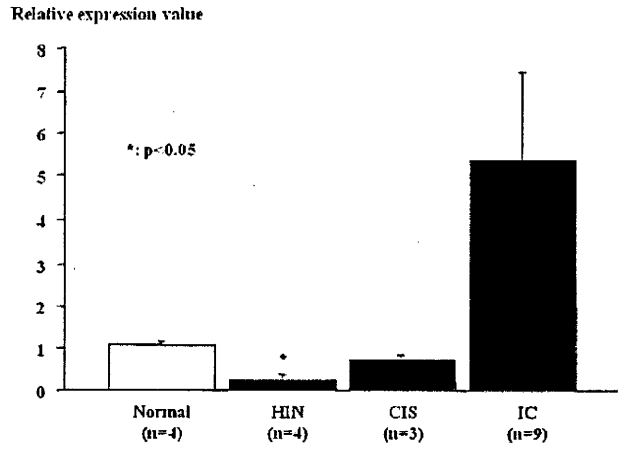

Figure 2. Quantitative RT-PCR analysis of miR-10a expression levels in the tumor samples and non-cancerous mucosa of the esophagus. The expression levels significantly decreased in high-grade intraepithelial neoplasms (HGIN) and tended to be low in non-invasive ESCC (carcinoma *in situ*, CIS), despite the results being insignificant. No difference was noted in the miR-10a expression levels between the invasive cancer (IC) phenotype and the other groups.

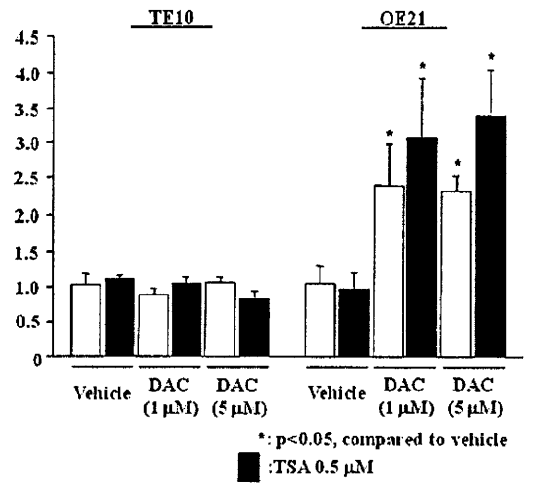

Figure 3. OE21 cells incubated with or without 5-aza-2'-deoxycytidine (DAC, 1 or 5 μmol/l) for 96 h, followed by treatment with 0.5 μmol/l trichostatin A (TSA) or vehicle for an additional 24 h. Total RNA was extracted and subjected to the quantitative RT-PCR to measure cellular miR-10a expression levels. Treatment with 1 μmol/l of DAC restored the miR-10a expression levels. TSA treatment did not affect miR-10a expression.

substantially down-regulated in ESCC cells compared to the Barrett's esophageal adenocarcinoma ones (Fig. 1D). These results indicate that miR-10a expression may be differentially down-regulated in SCC of the esophagus.

Down-regulation of microRNA-10a in esophageal high-grade intraepithelial neoplasm and non-invasive esophageal squamous cell carcinoma (carcinoma in situ). MiR-10a expression in ESCC tumor samples and non-cancerous tissues was assessed using real-time RT-PCR (Fig. 2). Relative miR-10a expression levels were significantly lower in esophageal HGIN and tended to be low in non-invasive ESCC (carcinoma *in situ*), which were histopathologically classified according to the guidelines of the Japanese Esophageal Society for the diagnosis and treatment of ESCC (17), compared to the non-tumor mucosa. MiR-10a expression was heightened in the invasive ESCCs despite the results being insignificant.

Restoration of microRNA-10a expression with 5-aza-2'-deoxycytidine treatment. Relative expression levels of miR-10a significantly increased in the presence of DAC, even at a concentration of 1 μmol/l in OE21 (Fig. 3). However, no increase was noted in the TE10 cells (data not shown). No significant effects on miR-10a expression were observed following incubation with TSA at a sufficient concentration in the cell lines (Fig. 3).

Discussion

In the present study, miR-10a was substantially down-regulated in ESCC cells. The miR-10a expression level was significantly lower in the ESCC cell lines examined compared to non-malignant esophageal squamous cells. In addition, miR-10a was substantially lower in the ESCC cells compared to those derived from esophageal adenocarcinoma, another significant type of esophageal cancer (2). Nevertheless, miR-10a expression did not necessarily decrease when compared with the remaining human malignant cell lines. These data implicate miR-10a in ESCC pathogenesis, and further functional analyses may shed light on the diagnostic and therapeutic potential of miR-10a against this malignant disease.

Down-regulation of miR-10a has been reported in chronic myeloid leukemia (CML) (18). Among 157 miRs tested using quantitative RT-PCR, miR-10a, along with miR-150 and miR-151, was listed in the significantly down-regulated miRs in CML cells compared to CD34-positive cells taken from healthy controls (18). The clinical relevance of this finding was shown in a group of 85 newly-diagnosed patients with CML in which the expression of miR-10a was down-regulated in 71% of cases (18). On the other hand, previous studies showed an overexpression of miR-10a in other cancers, including hepatocellular, pancreatic and urothelial carcinomas and acute myeloid leukemia (19-21). Based on the real-time PCR analysis and using RNA-extracted, formalin-fixed, paraffin-embedded, archival liver tissue, miR-10a expression levels were significantly increased in hepatitis C virus-associated hepatocarcinoma compared to normal liver parenchyma (19). Northern blot analysis showed increased expression levels of miR-10a in metastatic pancreatic adenocarcinoma (20). Weiss *et al* explored the metastatic behavior of primary pancreatic tumors and cancer cell lines in xenotransplantation experiments. These authors found that miR-10a expression promoted metastasis of the tumor cells, and the repression of this expression was sufficient to inhibit invasion and metastasis formation (20). The regulatory actions of miR-10a were mediated via its target inhibition of HOXB1 and HOXB3 expression (20), implying that miR-10a is a significant mediator of tumor metastasis. In the clinical settings, the present study showed a comparable down-regulation of miR-10a in HGIN and non-invasive ESCC, whereas it was highly expressed in the invasive ESCCs. The exact reasons for this discrepancy remain unknown, but the abundance of miR-10a expression may be involved in ESCC development and progression. There

may be differential miR-10a expression in human cancers including ESCC, which may affect cellular transformation, carcinogenesis and aggressive behavior and act as an oncogene or tumor suppressor (11,13).

Transcriptional deregulation, epigenetic alterations, mutations in miR sequences, DNA copy number abnormalities and dysfunction in their biogenesis machinery may contribute to the aberrant expression of miRs in human cancers (22), though the underlying mechanisms remain unknown. Recent evidence has shown that epigenetic changes, including DNA methylation and histone modification, play important roles in regulating expression of not only protein-coding genes but also certain miRs (22,23). In the present study, treatment with a demethylating agent restored miR-10a expression in OE21 cells. Han *et al* compared miR expression profiles between human colon cancer cell line HCT 116 and its derivative, DNA methyltransferase 1 and 3b double knockout (DKO) cells, and found that the expression of approximately 10% of miRs may be regulated by DNA methylation (23). Of note is that miR-10a was the most strikingly up-regulated miR in the DKO HCT116 cells. Additionally, well-defined CpG islands are located within 3 kb upstream of the miR-10a gene locus. Bisulfite sequencing showed that the majority of CpG sites proximal to miR-10a were hypermethylated in the parent cell line, while DNA methylation was largely absent in the DKO cells (23). Thus, epigenetic regulatory mechanisms may be involved in miR-10a expression, at least in certain human cancer cells.

In conclusion, based on the comprehensive microarray analysis, following quantitative confirmation with the real-time RT-PCR procedure, miR-10a expression was specifically down-regulated in ESCC cells. miR-10a expression was comparably low in HGIN and non-invasive ESCC cells, whereas the expression levels increased in the invasive phenotypes, suggesting unique regulatory mechanisms for the differential expression of miR-10a. In this context, miR-10a expression is likely to be regulated via DNA methylation in the CpG islands proximal to its gene locus in certain ESCC cells.

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シンポジウム－4

在外被爆者検診・健康相談事業の現況と展望

1）長崎大学病院　永井隆記念国際ヒバクシャ医療センター
2）大韓赤十字社　ソウル病院
3）日本赤十字社　長崎原爆病院

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長崎医学会雑誌85巻原爆特集号別冊
平成22年9月25日発行

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1)はじめに

在外被爆者とは，日本国外に居住する原爆被爆者を意味する．世界30カ国に約4300人であり，第２次世界大戦当時，広島・長崎にいて被爆した韓国の方や，戦後北米や南米などへ海外移住した日系人の方が大半を占めている．今日，日本政府は，海外に居住する被爆者に対しても，国籍を問わず被爆者健康手帳を交付している．しかし海外においては，被ばく医療に対する医療機関の理解は，長崎・広島に比較すると極めて低く，また必ずしも日本と同様な医療等の支援が受けられるとは限らない．そこで長崎大学病院においても，長崎原爆病院，大韓赤十字社，NASHIM，厚生労働省・長崎県・長崎市などと協力し，在外被爆者に対する支援事業を積極的に推進している．今回，韓国の原爆被爆者検診・健康相談事業を中心に，その現況と展望を紹介したい．

2)在外被爆者と支援事業の歴史的背景

在外被爆者は，厚生労働省のデータによれば2008年３月現在，韓国に約2900名，北米に約1000名，南米に約180名，世界30数カ国に総計約4300名余りが，在住している[1)]．在外被爆者検診は，1977年より隔年で北米健診が実施されており，2009年に第17回北米検診が行われた．南米健診は，1986年より隔年で実施されており，2010年秋に第13回検診が予定されている．いずれも広島県・広島医師会が中心となって行っている．韓国については，2004年～2010年までに長崎県・長崎大学・長崎原爆病院が中心となり計13回の在韓被爆者健康相談を実施し，本年秋に第14回健康相談が予定されている．

南米・北米を中心とした日系人の在外被爆者については，日本が貧しかった時代の戦前・戦後の移民政策を理解する必要がある．戦争被害がひどかった沖縄，広島，長崎などでは人口の割合に比して移民が多く，被爆者も移民として南米・北米へ渡られた方々がたくさんおられた．一方，台湾は日清戦争後の1895年，韓国は1910年に日本に併合された．この時代に，長崎や広島に在住したり，徴兵・徴用・留学などで来ていたりして被爆され，韓国や台湾などに戦後帰国された被爆者の方々がおられる．特に戦前，韓国から広島へ家族をともなって移住された方々は非常に多く，原爆被爆者の数も２～３万人余りから，記録によっては両市合わせて10万人に上るともいわれている．

戦後の被爆者支援事業は，初期は国内の日本人が中心で，在外被爆者の方々の支援は十分にはなされていなかった．広島・長崎の民間医療機関が中心となって継続されていた在外被爆者に対する支援に加え，1981年の日韓両国政府合意に基づき，５年間の公的支援入

院検査が，韓国人被爆者349名(広島226人，長崎123人)を対象に行われた．1991年～1993年に韓国の被爆者医療支援として，日本より総計40億円の拠出がなされた．また南米などより里帰り入院が，長崎・広島の原爆病院中心に受け入れられた．2003年より在外被爆者に対する本格的渡日治療が開始され，2004年よりはじめての本格的な韓国被爆者に対する健康相談事業が開始された．

3)在韓被爆者健康相談事業

(i)検診と健康相談の準備と実施状況

我々が行っている在韓被爆者検診及び健康相談事業は，日本における原爆健診精密検査に相当する検診を健康相談事業の3ヶ月前から1ヶ月前にかけて協定病院にて行っている．事前に担当病院への視察と検診協力お願いも行っている．具体的な検診項目は，身長・体重・視力・聴力検査，血液・生化学検査(腫瘍マーカーを含む)，尿検査，便潜血反応，胸部X線，腹部エコー，胃内視鏡(または上部消化管透視)，骨密度，子宮頸がん検診，マンモグラフィーなどである．検診から2週間から3週間後に，検診結果を郵送する．重大な異常が認められた場合は直接患者へ現地検診担当医療機関が連絡している．検診結果は韓国語で書かれているため，日本の専門医が相談しやすいように，検診結果の日本語訳を通訳と協力して行っている．

健康相談事業に参加可能な医師団(5～7名)を，長崎大学病院・長崎原爆病院・放射線影響研究所より推薦してもらい，長崎県・長崎市より行政相談可能なスタッフ(2名)と健康体操担当の保健師の人選(1名)を行う．健康相談のできる通訳の人選を行う．また健康相談事業の事前説明会を行い，よりよい相談事業ができるよう日本側と韓国側担当者の認識を共有できるように努めている．

健康相談事業は，5日間の日程で行われているが，大韓赤十字社のスタッフが，被爆者に対する連絡や相談会場の事前調整などを行ってくれており，また2日前より会場の整備や機材の搬入にあたってくれている．

健康相談当日は，健康診断結果票と問診票をもとに通訳を介して，我々が平均40分をめどに内科健康相談を行う．ここで診断や医学的問題点，希望する支援内容などをリストアップする．診断はなるべくICD-10準拠病名を記載するよう努める．様々な問題点を抱える患者の場合は相談が長時間に及ぶこともあり，1日の相談人数は約90名以下である．精査・治療などの至急対応が必要な場合は，協力病院の韓国人医師へ英文紹介状を書き，約30%の方々を紹介している．健康相談の結果表には，画像診断などは所見のみの記載のため，画像を直接見ないと判断できないケースは，読影できるように準備している．腰痛やひざ痛などを訴える被爆者は多いので，整形外科医の相談や理学療法士による指導，保健師による健康(腰痛)体操などのプログラムも用意している．

渡日治療や，原爆症認定などの行政相談の希望がある場合は，長崎県・市の被爆者援護課職員と大韓赤十字社職員がその相談にあたる．それらの途中で医学的な判断が必要な場合は，我々日本人医師に再度相談があることもある．

一方，心のケアが必要と判断される場合は，長崎大学精神科医または臨床心理士による心の健康相談も行っている．

尚，日本の原爆手帳を有していないが，韓国側の原爆被爆認定書を有している方が，少数であるが受診されるので，その方々の相談にも対応している．

(ii)これまでの実績及び在韓被爆者からの評価

2004年～2010年にかけて韓国内11か所で，年2回健康相談事業を行い，延べ2937名の在韓被爆者の健康相談を行った．男女比はほぼ1対1で，健康相談の場所と毎回の受診者数・平均年齢は以下のとおりである．

	実施時期	場 所	受診人数	平均年齢
第1回	2004年6月	陜川	70名	78
第2回	2004年10月	大田・平澤	60名	73
第3回	2005年6月	ソウル	297名	69
第4回	2005年10月	大邱	362名	68
第5回	2006年5月	陜川	322名	71

第6回	2006年11月	釜山	238名	68
第7回	2007年5月	馬山	171名	68
第8回	2007年11月	光州・済州島・大田・平澤	83名	73
第9回	2008年6月	ソウル・江原道	275名	70
第10回	2008年10月	大邱	304名	70
第11回	2009年9月	陜川	296名	73
第12回	2010年2月	釜山	278名	70
第13回	2010年5月	馬山	196名	69

検診の参加者の81%の方々は健康相談を受診されている．検査結果を事前に受け取られているのにもかかわらず，片道数時間かかっても相談を受けに来られた方々が沢山おられ，健康相談を心待ちにされていると感じられた．

大韓赤十字社が行ったアンケートによれば，受診者の被爆者健康相談への満足度は，第3回(ソウル)64.5% → 第4回(テグ)81.0% → 第5回(陜川)88.4% → 第6回(釜山)93.0% → 第7回(馬山)91.4% → 第9回(ソウル)95.9%と回を経るごとに満足度が上がっている．最近の相談においては，常に満足度95%前後とのことである．また被爆による健康不安の解消に，健康相談が役立ったかという質問項目も，90%前後の受診者が役立ったと答えている．

今回2005年と2008年のソウルにおける健康相談の結果について，悪性腫瘍の診断について比較してみた．2005年には，既往歴と検診で発見された分あわせて，延べ12(4.0%)の悪性腫瘍(内訳：胃癌2，大腸癌2，肺癌0，肝癌0，胆嚢癌0，甲状腺癌1，腎癌1，膀胱癌0，前立腺癌3，子宮癌0，悪性黒色腫0，悪性リンパ腫1)が診断された．一方，2008年には延べ26(9.5%)の悪性腫瘍(内訳：胃癌7，大腸癌5，肺癌1，肝細胞癌1，胆嚢癌1，甲状腺癌1，腎癌0，膀胱癌1，前立腺癌4，子宮癌1，悪性黒色腫1，悪性リンパ腫1)が診断されており，15腫瘍以上(5.5%以上)が2008年の検診を含め，新たに発見されたと考えられる．日本対がん協会の2006年がん検診のまとめで，被爆者検診と同様な内容の検診における癌の発見率が0.84%である[2]．一方，日本のがんの65-74歳の罹患率は年間1.4%程度であり[3]，3年間のがん罹患率は4.2%と考えられる．一概に比較することはできないが，韓国における被爆者検診＆健康相談は，疾患の早期発見に寄与していると考えられる．

(iii)被爆者のこころの健康

原爆被爆に伴う精神的影響や在韓被爆者の社会状況が，被爆者の方々の心の健康に影響を与えている可能性があるため，心の健康相談を行っている．日本の被爆者につては，本田らが被爆後50年の時点で，長崎の被爆者3526人を対象に調査した結果，GHQ12を用いた心の健康状態では，8.4%に4点以上の高スコア(心的ストレスが高い状況)が見られた[4]．韓国の被爆者は，社会環境が異なり直接は比較できないが，今回中根，越本らが行った373人の調査では50.5%にGHQ12の高スコアを認めた[5]．原爆に被災に伴う心的ストレスをIES-R質問票をもとに調査した結果では，IER-R高得点群は韓国の被爆者32.7%であり，本田らの調査における長崎被爆者28.4%と同等であった．長崎の被爆者においても，近距離被爆，急性被ばく症状の出現，近親者の死亡などの被爆体験は，社会・生活条件に加えて，心の健康状態に寄与していた．韓国の被爆者においても，まだ解析中であるが，GHQ12とIES-Rは相関しており，心の健康に被爆体験は重要な要素であると考えられた．一方，長崎の調査では，男女差による影響はなかったが，韓国では女性にGHQ12の高得点者が多く，男女差の社会的環境が異なることが推測された．

4)在外被爆者健康相談のまとめと展望

在外被爆者健康相談は，通常の健康な人を対象とした健診と異なり，様々な疾患をすでに抱えた被爆者を多く対象としていることより，個人個人の問題解決にそった相談を行っている．癌やその他の疾患の早期発見や早期治療が急激に増えているのは，これまで在韓被爆者の医療アクセスが十分でなかったことに加え，健康相談により自身の病状を把握してもらった教育効果もあると考えられる．

在外被爆者健康相談のバックアップ機能の一つとしての渡日治療について，長崎大学病

院では7年間で延べ157名の渡日治療入院患者を受け入れた．健康相談の担当医師が，渡日治療にも関わることは，治療効果を上げる意味でも，渡日治療のリスクを軽減する意味でも重要であると思われる．

在外被爆者に対する検診や健康相談による支援のあり方は，高齢化がさらに進むことにより，認知症をはじめとする老齢疾患の増加により，さらに複雑化してゆくと考えられる．海外における医療制度や特有の感染症を理解するといった一般的なことだけでなく，疾患構成や医療・社会状況の変化に応じた，健康相談内容の深化が必要とされる．長崎大学においては，現地医療機関，大学，行政機関，患者団体などと，健康相談時の限られた協力だけではなく，長崎・ヒバクシャ国際医療協力会(NASHIM)などを通じた，より緊密な被ばく医療ネットワーク構築を支援しているが，そのような多面的活動がいっそう重要となる．さらに在外被爆者においても，長期の発がんなどのリスクを心配することによる放射線災害特有の心的ストレスと，社会経済状況などによるスティグマを明らかにし，よりよい解決策を見出すことが，原爆だけでなくチェルノブイリ原発事故などの放射線災害の被災者の心の健康の問題の解決に向けて喫緊の課題となっている．

謝　　辞

本事業の中心的役割をはたされてきた熊谷敦史[1]，岩永正子[1,2,4]，塚崎邦弘[2]，越本莉香[1]，中根秀之[3]，古河隆二[5]，赤星正純[6]，OH Sang-eun[7]，([1]長崎大学病院　国際ヒバクシャ医療センター，長崎大学大学院　[2]原研内科，[3]保健学科，[4]活水大学，[5]長崎原爆病院，[6]放射線影響研究所，[7]大韓赤十字社，敬称略)，諸先生方に敬意を表したい．さらに長崎大学原研内科の宮崎教授，精神科の小澤教授には教室全体で支援いただいた．南米被爆者検診・健康相談事業は，広島県医師会の有田 健一先生，桑原 正雄先生，松村 誠先生にたいへんお世話になった．また検診や健康相談事業の様々な調整を行っていただいた行政・被爆者団体・大韓赤十字社スタッフ・通訳・ボランティアの方々の力なくしては，海外での被爆者支援活動が成り立たないことを強調し，あらためて感謝申し上げたい．

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J Gastroenterol
DOI 10.1007/s00535-010-0349-7

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Predictive value of tumor markers for hepatocarcinogenesis in patients with hepatitis C virus

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Received: 9 August 2010 / Accepted: 22 October 2010
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Abstract
Background Increases in tumor markers are sometimes seen in patients with chronic liver disease without hepatocellular carcinoma (HCC). The aim of this study was to determine the relationship between the levels of three tumor markers [alpha-fetoprotein (AFP), *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3%), and des-γ-carboxy prothrombin (DCP)] and hepatic carcinogenesis to identify hepatitis C virus (HCV) carriers at high risk for cancer development.
Methods A total of 623 consecutive HCV carriers with follow-up periods of >3 years were included. The average integration values were calculated from biochemical tests, and tumor markers, including AFP, AFP-L3%, and DCP, and factors associated with the cumulative incidence of HCC were analyzed.
Results HCC developed in 120 (19.3%) of the 623 patients. Age >65 years [adjusted relative risk, 2.303 (95% confidence interval, 1.551–3.418), $P < 0.001$], low platelet count [3.086 (1.997–4.768), $P < 0.001$], high aspartate aminotransferase value [3.001 (1.373–6.562), $P < 0.001$], high AFP level [≥10, <20 ng/mL: 2.814 (1.686–4.697), $P < 0.001$; ≥20 ng/mL: 3.405 (2.087–5.557), $P < 0.001$] compared to <10 ng/mL, and high AFP-L3% level [≥5, <10%: 2.494 (1.291–4.816), $P = 0.007$; ≥10%: 3.555 (1.609–7.858), $P < 0.001$] compared to <5% were significantly associated with an increased incidence of HCC on multivariate analysis.
Conclusions Increased AFP or AFP-L3% levels were significantly associated with an increased incidence of HCC. Among HCV carriers, patients with ≥10 ng/mL AFP or patients with ≥5% AFP-L3% are at very high risk for the development of HCC even if AFP is less than 20 ng/mL or AFP-L3% is less than 10%, which are the most commonly reported cutoff values.

Keywords Alpha-fetoprotein (AFP) · *Lens culinaris* agglutinin-reactive fraction of AFP · Hepatic regeneration · Necroinflammatory activity · Hepatocarcinogenesis

Introduction

Serum alpha-fetoprotein (AFP) is a widely used marker for hepatocellular carcinoma (HCC) [1]. However, serum AFP levels are increased in patients with liver diseases other than HCC, including viral hepatitis [2–4], with a prevalence of 10–42% [2, 5–7]. Increases in AFP are a marker of hepatic regeneration following hepatocyte destruction in viral hepatitis [8]. However, the pathogenesis and clinical significance of this phenomenon remain unclear.

The *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3%) and des-γ-carboxy prothrombin (DCP) are also markers for HCC [9–12]. Available data suggest that these tumor markers are more highly specific for HCC than AFP alone [9]. However, there are no reports examining the prognostic value of these markers in hepatocarcinogenesis.

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Published online: 07 December 2010

Results of biochemical tests, including tumor markers, can fluctuate for a given patient and can vary between different patients, and repeated measurements over time may provide a more accurate picture of disease development or progression. The arithmetic mean value is often used to assess biochemical parameters over time, but this value can be greatly affected by the interval between measurements such that a short period of very high values can inappropriately skew the mean. We have previously argued that the average integration value is more meaningful than the arithmetic mean value for the purposes of monitoring disease progression [13, 14].

The aim of this study was to determine the relationship between three tumor markers (AFP, AFP-L3%, and DCP) to better identify hepatitis C virus (HCV) carriers at high risk for the development of HCC. Of note, we used the average integration values of these parameters in our analysis.

Patients, materials, and methods

Patient selection

A total of 1623 consecutive patients positive for anti-HCV antibody visiting the Department of Gastroenterology at Ogaki Municipal Hospital during the period January 1995 to December 1997 were considered for enrollment. The present study cohort included the following criteria for enrollment: (1) positive for anti-HCV antibody by second- or third-generation enzyme-linked immunosorbent assay and detectable HCV RNA for at least 6 months; (2) no evidence of positivity for hepatitis B surface antigen; (3) exclusion of other causes of chronic liver disease (i.e., alcohol consumption lower than 80 g/day, no history of hepatotoxic drug use, and negative tests for autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease); (4) follow-up period greater than 3 years; (5) measurement of AFP, AFP-L3%, and DCP at least every 6 months; (6) no evidence of HCC for at least 3 years from the start of the observation periods; and (7) interferon (IFN) therapy completed greater than 3 years before the detection of HCC in patients who received IFN therapy. A total of 623 patients fulfilled these criteria (Fig. 1).

Fibrosis was histologically evaluated in 187 of the 623 patients and staged according to Desmet et al. [15] as follows: F0, no fibrosis; F1, mild fibrosis; F2, moderate fibrosis; F3, severe fibrosis; and F4, cirrhosis. The remaining 436 patients were evaluated by ultrasound (US) findings and biochemical tests. The diagnosis of cirrhosis was made according to typical US findings, e.g., superficial nodularity, a coarse parenchymal echo pattern, and signs of portal hypertension (splenomegaly >120 mm, dilated portal vein diameter >12 mm, patent collateral veins, or ascites) [16–18]. In this study patients who did not satisfy these criteria were classified as having chronic hepatitis. Four hundred and sixty-three patients were diagnosed with chronic hepatitis and 160 patients with cirrhosis.

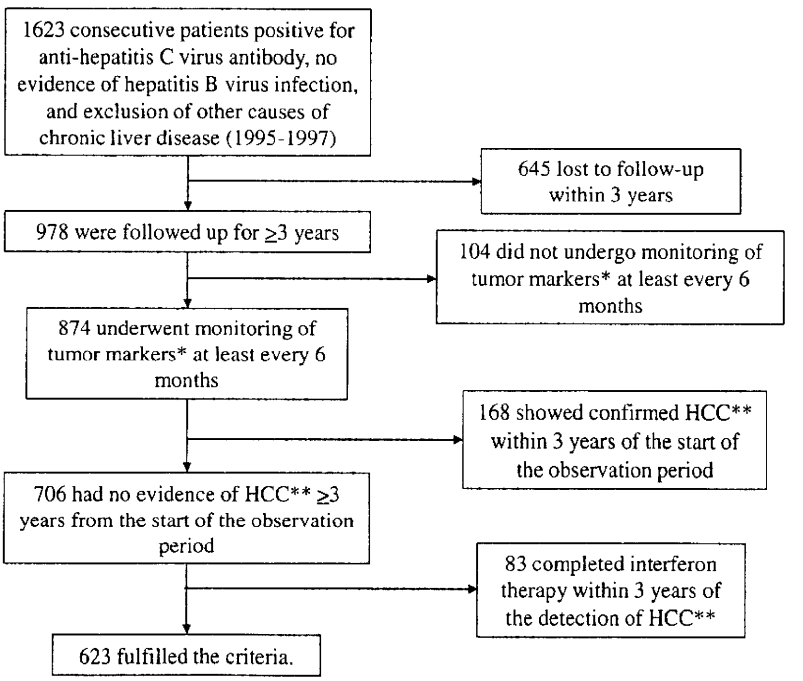

Fig. 1 Schematic flowchart of enrolled patients. *Serum alpha-fetoprotein (AFP), *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3%), and des-γ-carboxy prothrombin (DCP). **Hepatocellular carcinoma (*HCC*)