III. MATERIALS AND METHODS

1. Specimen collection and storage

In this study, we collected tissues of cancer patient with a total number of 114, which include 76 colorectal cancer patients, 20 liver cancer patients and 18 lung cancer patients who had undergone surgical resections for primary cancer at Maharaj Nakorn Chiang Mai Hospital during April 2003 to June 2004. In each case, normal tissues were collected together with the corresponding tumor tissues for comparison. These tissues were immediately placed in vials, frozen in embedded medium for the preservation of cell integrity, and stored at -70 °C until analyzed. They were classified by a pathologist according to pathological features of the tumors, which included tumor size in maximal diameter, depth of invasion, venous invasion, lymphatic invasion, perineural invasion, histological grading, lymph node metastasis, distant metastasis, and tumor staging (the AJCC TNM classification). Clinical history of each patient were searched from the outpatient department (OPD) card at the administration and clerical section, Maharaj Nakorn Chiang Mai Hospital. The study processes were thoroughly accepted by the ethical committee of the Faculty of Medicine, Chiang Mai University according to document number 56/2545.

Serums were collected from 77 cancer patients prior to the operation and from 52 healthy volunteers to be subjected for comparison. All serum were stored at -70 °C until analyze.

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2. Preparation of tissue homogenate

Tissue was thawed at room temperature (RT), five volumes (w/v) of 69 mM SDS lysis buffer (see Appendix), containing protease inhibitors (Roch, Germany) of the weighed (0.08-0.3 g). For example, if tissue weigh 100 μg, 500 μl of lysis buffer was added. The sample was then homogenized (Eberbach, USA) using a bench-top homogenizer until it became homogeneous and immediately incubated at 95°C for 10 minutes to activate the denaturing activity of SDS. The resultant homogenate was then centrifuged at 15,000 x g at 4 °C for 15 minutes to remove

the cell debris. The supernatant was removed and total protein concentration in each tissue homogenate sample was measured. This sample was then frozen at –70 °C until used.

3. Estimation of protein concentration in serum and tissue homogenate by the bicichoninic acid (BCA) assay kit

Protein concentration was estimated using BCA protein assay kit purchased from PIERCE, which based on the method developed by Smith in 1985 (Smith *et al.*, 1985). The principle of this method is based on reduction of cupric cation (Cu^{2+}) to cuprous cation (Cu^{1+}) by protein (peptide bonds) in an alkaline medium (the biuret reaction) with the highly sensitivity and selective colorimetric detection of the cuprous cation using reagent containing BCA. The purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentration over a broad working range of 0.02-2 mg/ml.

3.1 Preparation of BSA (bovine serum albumin) standard

The 2.0 mg/ml BSA stock standard protein was diluted with dH_2O to various concentrations for preparing protein standard curve, which described below in order to determine the protein concentration of unknown samples.

Volume of stock standard (µl)	Volume of dH_2O (µl)	Final concentration (mg/ml)
400	0	2
240	160	1.2
200	200	1.0
160	240	0.8
120	280	0.6
80	320	0.4
40	360	0.2
0	400	0

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3.2 Preparation of BCA working reagent

Prior to the assay, fresh working reagent was prepared by mixing 50 parts of reagent A (bicinchoninic acid) with 1 part of reagent B (4% cupric sulfate). An appropriate amount for 96-well plate detection was 20 ml of reagent A combined with 0.4 ml reagent B. After combinding, this working reagent was stable at RT for at least one day.

3.3 Protein measurement protocol using microplate

Serum and tissue homogenate was diluted 1:100 and 1:10, respectively, with water if necessary to achieve concentration within the linear range of standard curve. Ten microlitres of each sample was added in quadruplicate to a 96-well plate together with BSA standard protein ranging from 0.2-2 mg/ml. Then, 190 μl of the working reagent was added to each well. The plate was covered with cling film and incubated at RT for 30 min. After incubation, the absorbance was measured at 550 nm on a microplate reader (Bio Kinetics Reader EL340, USA). The average absorbance of all samples was determined and a protein concentration was estimated from standard curve. The measured protein concentrations were then used to calculate a volume of serum and tissue homogenate needed for gel electrophoresis (25 μg).

4. Assessment of tissue VEGF and circulating VEGF protein expression by Western blotting

Western blot is technique for the identification and quantitation of protein. This technique depends on the electrophoretic transfer of proteins from the gels to membrane and blotted proteins on an exposed surface is blocked by blocking solution (skimmed milk), to prevent unoccupied protein binding site from non-specific binding of antibody. The blocked blot is then detected the target protein by antibody probing. The specific primary antibody is used to detect the protein of interest, while the secondary antibody is directed against the primary antibody and conjugated with the enzymes, that is horseradish peroxidase (HRP) that allow visualization. Following binding and washing, detection substrates are applied to visualize detected protein.

The principle of chemiluminescent system, enhanced chemiluminescence (ECL), bases on the detection of light emission that is achieved by performing the oxidation of luminol by the HRP in the presence of chemical enhancers such as phenols. The oxidation of luminol in

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alkaline conditions effects the luninol in an excited state, which then decays to ground state via a light emitting pathway. The light produced by this ECL reaction peaks after 5-20 min and decays slowly thereafter with a half-life of approximately 60 min. The maximum light emission wavelength is at 430 nm, which can be detected by a short exposure to blue light sensitive autoradiography film.

4.1 Separation of protein by SDS-PAGE

SDS-PAGE is the most widely used technique for quantification of complex mixtures of proteins (Laemmli, 1970). In native form, proteins fold into complex secondary, tertiary, and quaternary structures. Their surfaces may be hydrophobic or hydrophilic, with greater or lesser distribution of charge and reactive groups. The rate of migration of native proteins through a sieving medium depends on their shapes and molecular weight. SDS-PAGE is a technique that denatures the proteins to nullify structural effects on mobility by SDS, denaturant, allowing separation on a ratio of a charge to mass basis. They also separate subunits from multimeric proteins by 2-mercaptoethanol (2-ME), which is a reducing agent to disrupt any disulfide bonds through reduction. SDS is an anionic detergent. It denatures proteins by binding to the protein chain with its negative charge molecule, dodecylsulfate group. A level of 1.4 g SDS binds to 1 g protein. This creates a charge to mass ratio that is consistent between proteins. Thus, separation on a SDS-PAGE occurs by mass alone.

The main principle of this method bases on the separation of proteins according to molecular size, as analyzing protein mixtures move through porous structure of polyacrylamide matrix. Polyacrylamide gels are formed from the polymerization of acrylamide monomer in aqueous solution in the presence of small amounts of a crosslinker, *N*, *N'*-methylenebisacrylamide (bis). Methylene group of bis is linked with two acrylamide molecules. The co-polymerization of them produces a cross-linked matrix that is a mesh-like network. The polymerization proceeds via a free-radical catalysis and is initiated by the addition of ammonium persulfate and the tertiary aliphatic amine *N*, *N*, *N'*, *N'*-tetramethylethylenediamine (TEMED). TEMED catalyzes the decomposition of the persulfate ion to produce a free radical. In addition, the pore size with polyacrylamide gel can be alternated by the total concentration and the ratio of acrylamide to bis.

Polyacrylamide gel is divided into an upper stacking gel and a lower running gel. The stacking gel has a large pore size, 2-4% acrylamide, which allows the proteins to move

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freely, and concentrate under the effect of the electric field. The running gel has a smaller pore, which can be altered in reproducible manner depending on size of analyzing protein. The recommended acrylamide concentrations for separation of different ranges of protein size are shown below.

Separation size range (kDa)	% Acrylamide in running gel
36 – 205	5%
24 – 205	7.5%
14 – 205	10%
14 – 16	12.5%
14 – 45	15%

4.1.1 Preparation of running gel (10% and 15%)

Two clean glass plates and spacer previously washed with water and ethanol were assembled and locked onto the gel-casting stand (Scie Plus, UK). The running gel solution was prepared following the method showed in 2.1 and 2.2 of Appendix C. The mixture was poured carefully into the slab mold until reached a level that leave the space for the comb to be inserted and 1 cm below the bottom of the wells. To avoid the meniscus effect, a thin layer of water is layered on the surface of the gel mixture before it polymerized. The mixture was allowed to polymerize at RT for 45 min or until the line between water and the top of the gel become visible. In this study, 10% and 15% running gel were used for the separation from protein of serum and tissues, respectively.

4.1.2 Preparation of stacking gel (4%)

After polymerization, over layered water was poured off and the stacking gel mixture was prepared (see Appendix C section 2.3). The solution was poured slowly onto the polymerized running gel until reached the top of the plates. Then, comb was inserted into the layer of the stacking gel solution and allowed to polymerize at RT for 45 min.

4.1.3 Preparation of the sample and electrophoresis procedure

The set of slab clamp with a solidified gel was removed from the casting stand and placed in the gel tank. The running buffer (see Appendix C section 1.8) was filled into the upper and lower chamber with a necessary volume of buffer. Ensure that the sample wells were fully filled with buffer. Then, the comb was removed by pulling straighten up slowly to

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avoid any damage of the well. After working out the amount of tissue homogenate needed and mixed with sample buffer as described previously, the protein mixture was heated at 95 °C for 5 min. First lane on each gel was loaded with 5 μl of molecular weight marker of protein (Fermentas, UK), while 25 μg protein of each sample mixture was applied onto the other lanes. Following application, the gel was run at 190 volts of constant voltage untill the bromphenol blue tracking dye reached the bottom of the running gel (1-1.5 hr) which electrophoresis was terminated.

After electrophoresis, the running buffer was discarded and the gel apparatus was dismantled. The glass-plate sandwich was pried open to remove the gel that was placed in the transfer buffer for further the blotting step.

4.2 Transfer of separated proteins

While the gel was running, two pieces of fiber pad, one piece of polyvinylidene fluoride (PVDF) membrane (8x6 cm in size, Pall corporation, UK) and two pieces of filter paper (9x7 cm in size) were soaked in transfer buffer (see Appendix C 3.1). Once the bromphenol blue had reached the bottom, the gel was removed from the electrophoresis tank. The following items were assembled in order starting from the black side of the cassette: fiber pad, filter paper, gel, membrane, filter paper, fiber pad, and white cassette clamp, respectively. The glass rod was used when necessary to smooth out any air bubble. The assembly was placed in the transfer tank with orientation of the black cassette closest to the negative electrode. Then, the transfer buffer was filled in the tank until reached the maximum filled lines. Electroblotting was performed by applying 30 volts at constant voltage for overnight and the water-cooled base of the tank was rinsed thoroughly with water to prevent overheating effects of the system. After transfer, the transfer sandwich was dismantled and then the blotted membrane was removed and placed in TBS-Tween buffer at 4 °C until detected.

4.3 Immunodetection

The blotted membrane was placed in plastic container and incubated with 20 ml of 2.5% skimmed milk in TBS-Tween for 1 hr at RT on a shaker (Gemmy VRN-200, Germany) to block non-specific binding sites. Primary antibody, rabbit anti-VEGF polyclonal antibody (Santa Cruze Biotechnology Inc., USA), was diluted (1:500) in 2.5% skimmed milk in TBS-Tween. Then, the blocking buffer was poured off and the blot was incubated with primary antibody for 2 hr at RT on a shaker followed by washing with seven changes of TBS-Tween

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each for 20 min. Secondary antibody, anti-rabbit immunoglobulins antibody conjugated with HRP (Dako, Denmark) was diluted (1:1,000) in 2.5% skimmed milk in TBS-Tween. After washing membrane, the blot was incubated for 2 hr at RT on a shaker with 8 ml of diluted secondary antibody then washed with six changes of TBS-Tween each for 20 min.

4.4 Visualization of the detected protein band

Prior to the detection, ECL Plus detection reagent (Amersham Biosciences, UK) was prepared by combinding solution A and B together with the ratio of 40:1. Sufficient volume to cover 8x5 cm membrane is 2.5 ml, which can be prepared by mixing 3 ml of solution A with 75 µl of solution B. The excess TBS-Tween was drained off from the washed membrane by touching the edge against a tissue paper and the membrane was placed on the piece of cling film with protein sided up. Then, the mixed detection solution was overlayered onto the protein side of the membrane and incubated for 5 min at RT, after which it was drained off. Then, the membrane was placed onto a new piece of cling film, protein sided down, wrapped up and gently smoothed out any air bubbles. The wrapped membrane was placed in the film cassette; protein sided up. In the dark room, a sheet of X-ray film (Eastman Kodak, USA) was placed on top of the membrane and the cassette was closed and allowed exposion for 30 sec-1 min. The exposure was justified according to the previous obtained signal to get optimal detection. After exposure, the film was developed by soaking in developing solution for 1 min (Eastman Kodak, USA), washing with dH_2O for 1 min. The developed film was then fixed in fixing solution (Eastman Kodak, USA) for 5 min, after which it was washed in dH_2O for 5 min, and stood at RT until dried.

4.5 Quantification of proteins on polyacrylamide gels by staining with coomassie blue

Proteins may be quantified in polyacrylamide gel after electrophoresis by staining with Coomassie Blue, which can provide an estimate of total amount of protein in each sample loaded onto a gel (Draber, 1991; Henkel and Bieger, 1994). These methods quantify total protein present but not one of protein in a mixture of several. The polyacrylamide gel carrying separated protein was submerged into generous amount of coomassie Blue staining solution (see Appendix C section 4.1) and incubate at RT, overnight. Next morning, the stained gel was de-stained by replacing coommassie blue staining solution with the de-stained solution I (see Appendix C section 4.2), and shaked slowly fro 30 min. This removed the buck of the excess

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stain. The de-stained solution I was replaced with de-stained solution II (see Appendix C section 4.3) and the solution was periodically until the gel background was clear.

5. Principle of Capture ELISA

An antibody specific for the substance to be measured is first coated onto a high-capacity protein binding microtiter plate. Any vacant binding sites on the plate are then blocked with the use of an irrelevant protein such as fetal calf serum (FCS) or bovine serum albumin (BSA). The samples, standards, and controls are then incubated on the plate, binding to the capture antibody. The bound sample can be detected using a secondary antibody recognizing a different epitope on the sample molecule, thus creating the "sandwich". The detection antibody is commonly direct conjugated to biotin, allowing an amplification procedure to be carried out with the use of streptavidin bound to the enzyme hoseradish peroxidase (HRP). As streptavidin is a tetrameric protein, binding four biotin molecules, the threshold of detection is greatly enhanced. The addition of a suitable substrate such as 3,3, 5,5-tetramethylbenzidine (TMB) allows a colormetric reaction to occur in the presence of the HRP that can be read on a spectrophotometer with the resulting optical density (OD) relating directly to the amount of antigen present within the sample.

5.1 Optimization of Capture ELISA

The objective of this step is to determine optimal condition for capture ELISA in order to measure VEGF level. There are many parameters which influence the result obtained in an ELISA. One of the most important parameters is antibodies concentration. The best way to determine the optimal capture and detection antibody concentrations is to perform a grid experiment. To from the grid, a 96-well plate was divided into 4 quadrants. Each quadrant is a minigrid, identifying different capture antibody and standard concentration at one particular detection antibody concentration. The 6 columns in each quadrant represented each concentration of capture antibody. As shown in Figure 3, the grid assay was performed by using mouse anti-VEGF monoclonal antibody (cat.no. MAB293, R&D system, USA) as a capture antibody and goat anti-VEGF polyclonal antibody as a detection antibody (cat.no. AF-293-NA, R&D system, USA). The capture antibody at concentration 0.25 μg/ml (row A-H, column 1, 2, 7, and 8), 0.5 μg/ml (row A-H, column 3, 4, 9, and 10), and 1 μg/ml (row A-H, column 5, 6,

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11, and 12) were coated into 96-well plate and incubated at RT for overnight. Coated plate was washed 5 times with 200 μl/well of washing buffer (PBS/0.05% Tween). After that, coated plated was block with 300 μl/well of blocking solution (1.5% skimmed milk) and incubated at RT for 2 hr. Standard recombinant human $VEGF_{165}$ were diluted with PBS containing 5% bovine serum albumin (BSA) from stock concentration 10,000 pg/ml (R&D System, USA) into 250, 500 and 1000 pg/ml and 100 μl of each concentration was applied into a 96-well plate as follows: 0 pg/ml (row A and E, column 1-12), 250 pg/ml (row B and F, column 1-12), 500 pg/ml (row C and G, column 1-12), and 1,000 pg/ml (row D and H, column 1-12). Then the plated was incubated at RT for 2 hr. After washing, different concentrations (50, 100, 200, 400 ng/ml) of detection antibody were applied (100 μl/well) and the plate was incubated at RT for 1 hr. Consequently, the plate was washed for 5 times and the HRP-conjugated anti-goat immunoglobulin (Dako, USA) at concentration 0.5 μg/ml was added and the plate was incubated at RT for 30 minutes. After extensive washing, 100 μl of TMB substrate solution was applied into each well and incubated in the dark for 20 minutes at RT, before the reaction was stopped by adding 50 μl of stop solution (H_2SO_4) (see Appendix C section 5.5) to each well. Optical density at 450 nm of each well was determined within 30 minutes, using a microplate reader.

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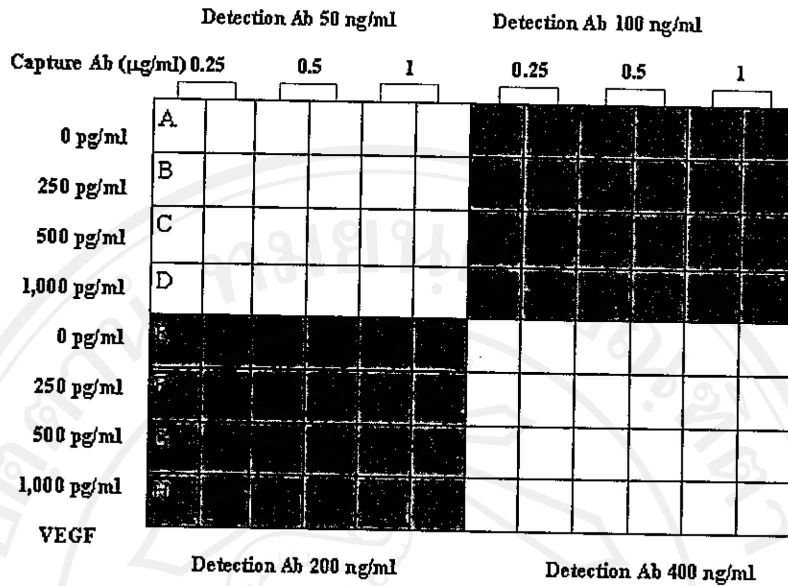

Figure 3. Checkerboard of assay optimization utilize mouse anti-VEGF monoclonal Ab (cat. no. MAB293) as a capture Ab and goat anti-VEGF polyclonal Ab (cat no. AF-293-NA) as a detection Ab

A grid experiment was also performed using goat anti-VEGF polyclonal antibody (cat.no. AF-293-NA, R&D system, USA) at concentrations 100, 200, and 400 ng/ml as a capture antibody and mouse anti-VEGF monoclonal antibody (cat. no. MAB293, R&D system, USA) at concentration 0.125, 0.25, 0.5, and 1 µg/ml as detection antibody, as shown in Figure 4.

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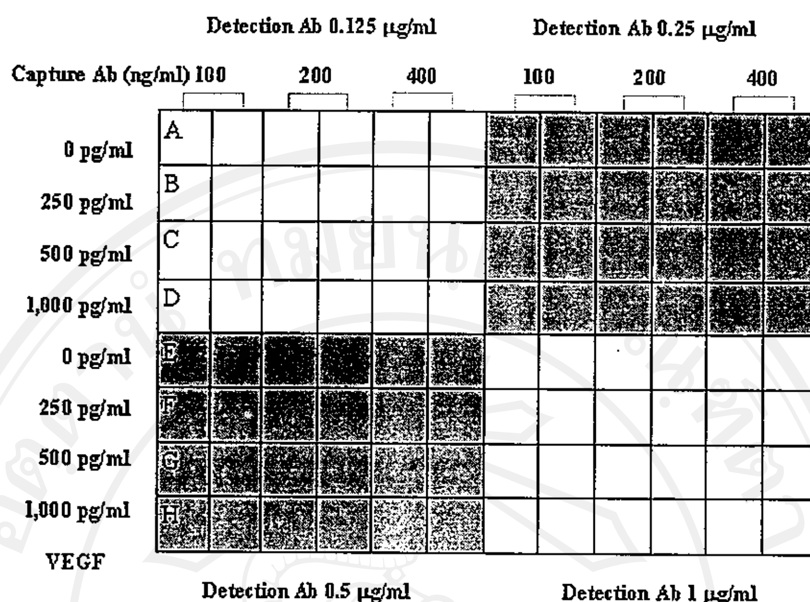

Figure 4. Checkerboard of assay optimization use goat anti-VEGF polyclonal Ab (cat. no. AF-293-NA) as a capture Ab and mouse anti-VEGF monoclonal Ab (cat. no. MAB293) as a detection Ab

5.2 Capture ELISA procedure

ELISA assay was performed to detect the tissues VEGF protein levels and serum VEGF level. ELISA wells were coated with 100 μl/well of goat anti-VEGF polyclonal antibody (200 ng/ml) (R&D system, USA) in PBS (see Appendix C section 5.2) and incubate at RT, overnight. After that, the plate was washed five times each with 200 μl/well washing buffer (PBS/0.05% Tween). Recombinant human $VEGF_{165}$ protein standard (stock concentration 10,000 pg/ml; R&D system, USA) was diluted in PBS containing 5% BSA into various concentrations (78.13, 156.25, 312.5, 625, 1,250, and 2,500 pg/ml). 100 μl of standard and unknown sample were added into each well and the plated was incubated at RT for 2 hr. After 5 washes with washing buffer, 100 μl of mouse anti-VEGF monoclonal antibody (0.5 μg/ml) was applied into each well and the plate was incubated at RT for 1 hr. The plated was washed for 5 times before 100 μl of goat anti-mouse IgG conjugated HRP (1:2,000) (Dako, Denmark) was applied into each well. After 30 minutes incubation and 5 washes, the HRP-conjugated anti-goat immunoglobulin (Dako, USA) at concentration of 0.5 μg/ml was added and the plate was

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incubated at RT for 30 minutes. After extensive washing, 100 μl of TMB substrate solution was applied into each well and incubated in the dark for 20 minutes at RT, before the reaction was stopped by adding 50 μl of stop solution (H_2SO_4) to each well. Optical density at 450 nm of each well was determined within 30 minutes, using a microplate reader.

5.3 Determination of the percentage of recovery of capture ELISA

A recovery study determines the ability of a test to measure a known incremental amount of standard analyte from a sample matrix. Thus, in practice, a known amount of recombinant human VEGF protein standard (A) is added to serum base (B) and subjected to capture ELISA (as shown in Table 4). After that, the recovery (C) is calculated as a concentration after performing the assay. The percentage of recovery is as follows:

$$\% \text{ Recovery} = (C-B)/A \times 100$$

Table 4. The preparations of serum sample for determination of the percentage of recovery

No.	Volume of serum (μl)	Volume of std VEGF (μl)*	Volume of diluent (μl)	Total volume (μl)	Concentration of VEGF (pg/ml)
1	450	-	50	500	0
2	450	5	45	500	500
3	450	10	40	500	1,000

* Std. Recombinant VEGF concentration 500 pg/100 μl

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6. Statistical analysis

Total VEGF levels are expressed as mean ± SD. Differences in the circulating VEGF levels of two independent groups were evaluated using the independent-sample t-test, whereas difference in the tissue VEGF level between normal and tumor tissues were analyzed using paired sample t-test. Correlating between VEGF isoform expression and the pathological features were evaluated using Chi-square test. All the statistical evaluations were performed by using the SPSS for Windows 10.0 package.

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