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Invited Paper

A novel 80kDa protein-tyrosin kinase in a human anaplastic large cell lymphoma with t(2;5)(p23;q35)

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We recently identified in a Ki-1 lymphoma cell line AMS3 a novel chimeric protein which is encoded on a chimeric gene locating partially on chromosome number 2 and the other part on 5 which is situated at the very translocation site. We identified partial sequences of this protein[1] and found that our protein is just the product of a newly found chimeric gene reported by Morris et al[2]. They identified this chimeric gene by the use of molecular method, positional cloning namely, while we reached this protein by the introduction of protein chemistry, mainly with the kinase assay. I would like to speak a little bit about the chemical specificities of this protein.

First, we established a Ki-1 lymphoma cell line, termed as AMS3, by the serial engraftment of biopsied lymphoma tissues into mice with severe combined immune deficiency (SCID mice)(Fig.1)[3]. Succeeding cytogenetic sudy revealed the cell line to carry a chromosomal translocation t(2;5)(p23;q35). Since previous reports have shown that this specific chromosomal translocation is rather specific on this type of lymphomas, we were so tempted to reveal what is happening around this translocation. This temptation was based on the knowledge that a large part of human malingnant lymphomas are caused by some abnormal expression of specific proteins (gene products) attributable to some chromosomal translocation. Abnormal expression of c-Myc protein on B-cell lymphomas

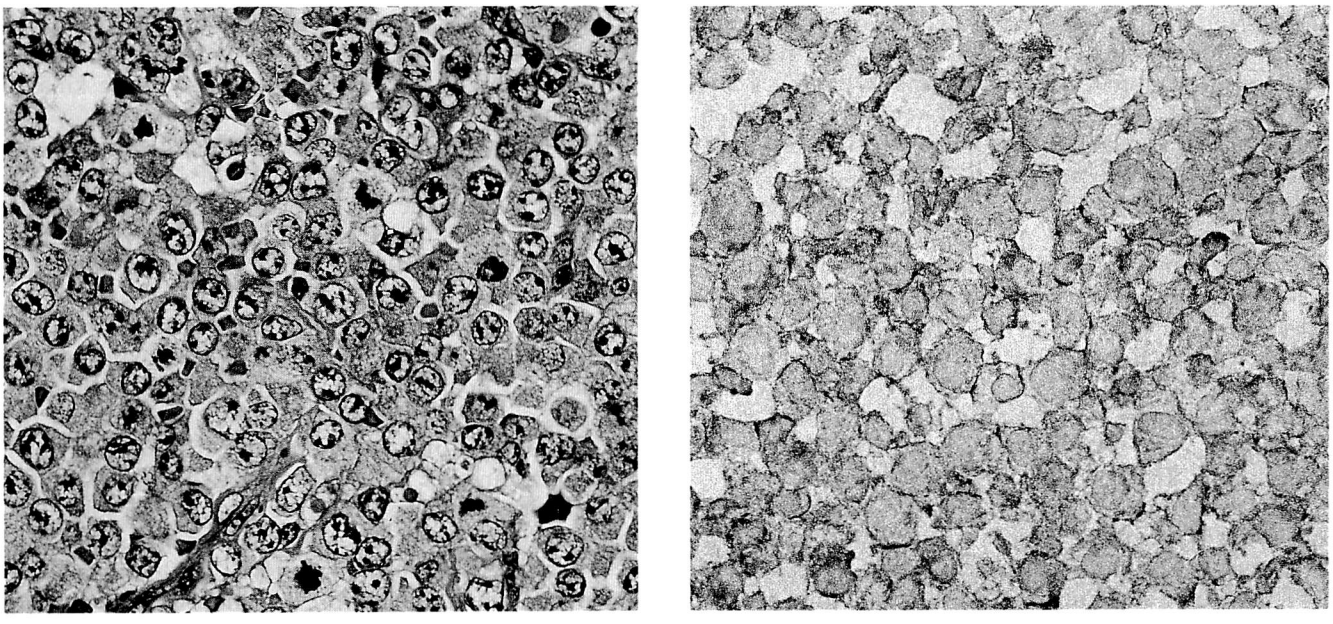

Fig.1. Histological studies of anaplastic large cell lymphoma (ALCL) case (a and b). a: Hematoxylin-Eosin staining of the originl biopsy specimen, showing large round nuclei and eosinophilic cytoplasm, typical ALCL cells. b: Immunostaining with CD30 (DAKO-BerH2) showing a positive reaction in most of tumor cells.

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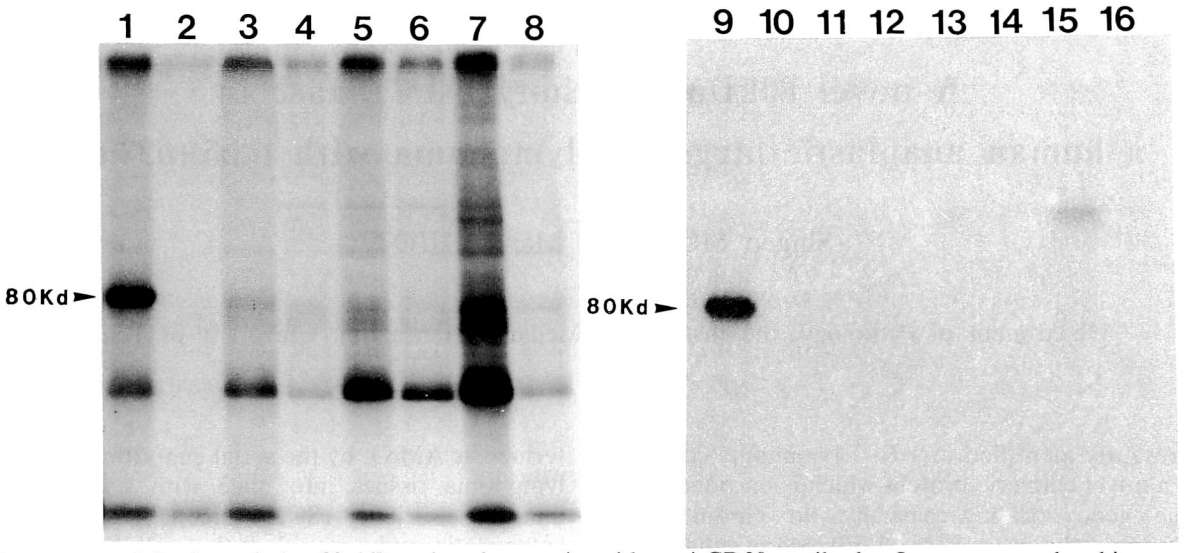

Fig.2. Immunoprecipitation of the 80 kDa phosphoprotein with anti-CD30 antibody. Immune-complex kinase assay. Immunoprecipitates with CD30 (RSC1) from following cell lysates were subjected to kinase assay: ALCL cells (lanes 1, 9), PBMC from healthy donor (lanes2, 10), PHA-P stimulated PBMC from healthy donor (lanes3, 11), CEM (lanes 4, 12), Jurkat (lanes 5, 13), MT1 (lanes 6, 14), Molt4 (lanes 7, 15) and SMS1, a B-cell lymphoma maintained in SCID mice (lanes 8, 16). Lanes 9-16 were treated with 1M KOH. The position of the 80 kDa protein is indicated.

caused by translocation t(8;14)(q24;q32) or Bcl-2 by t(14;18)(q32;q21) are the example of this fascination.

We started this heavy work by two methods, the one through protein chemistry and the other by genomic positional cloning (just as Morris et al. did). In the former study, we introduced kinase assay first. This is a kind of routine method for the detection of abnormal protein related to some phosphokinase reaction occuring intracellularly. Thus, we made lysate of this cell line, and immunocoprecipitated by anti-CD30 antibody BerH2 (Dakopatts). The precipitates were then electrophoresed and finally submitted for kinase assay by the addition of isotope-labeled phosphorus as the substrate. With these procedures, we could find a high expression of isotope-labeled phosphorus as the substrate. With these procedures, we could find a high expression of hitherto undescribed 80kDa protein phosphokinase, now termed as p80, that is quite unique on this specific cell line (Fig. 2). The succeeding phosphoaminoacid analysis revealed this unique protein to be the protein tyrosine kinase and not the serine or threonine kinases.

The next step was to clarify its peptide sequences. For this study, we had to obtain considerable amount of purified p80. The immunoprecipitation with anti-CD30 was found to be somehow unstable and the final gain of immunoprecipitate remained low. Thus, we now introduced anti-phosphotyrosine antibody (25.2G4, kindly gifted from Dr. Yamori T). This antibody could immunopercipitate p80 stably, permitting us to obtain proteins enough for peptide sequencing.

We collected the p80 in this way. The p80 was then digested with trypsin. Proteolytic fragments were then separated by HPLC(Fig.3-a). Six peaks were selected from this HPLC profiles, and they were applied diretly to an automated gas-phase microsequencer (Applied Bio)after re-chromatography. When the sequences of each peaks were obtained, they were submitted for the homology study. Four of these peptides were revealed to have homology with another protein tyrosine kinase LTK, while the remaining two were found not to have 70% homologies with any known proteins. Thus the p80 was shown to be a novel protein tyrosine kinase (Fig. 3-b, c).

After the submission and acceptance of this protein to certain Journal, the publication of Morris et al[2]. appeared in Science. We found that their cDNA sequences are identical to our p80. Now it became apparent that p80 is the very product of this chimeric mRNA.

The next step we did was to obtain specific antibody for p80. The antibody, if obtained was expected to produce big coutributions in revealing biological functions, as well as in diagnosis and clarification of specific subtype of Ki-1 lymphoma that express this unique protein. We did it by immunizing rabbits with the synthetic peptides whose sequences were followed by the above methods. The immune sera obtained from these rabbits were purified by $(NH_4)_2SO_4$ sedimentation and columun immunoprecipitation with the use of synthetic peptides as antigen. The purified antibody thus obtained (anti-p80) immunoblotted p80 from AMS3 lysates specifically. Also, this antibody could immunostain unfixed-frozen sections of AMS3 tumor obtained from SCID mice, as well as its original (biopsy) specimen[4]. This information promoted us to study other Ki-1 lymphoma tissues.

Now we collected 10 Ki-1 lymphomas, including

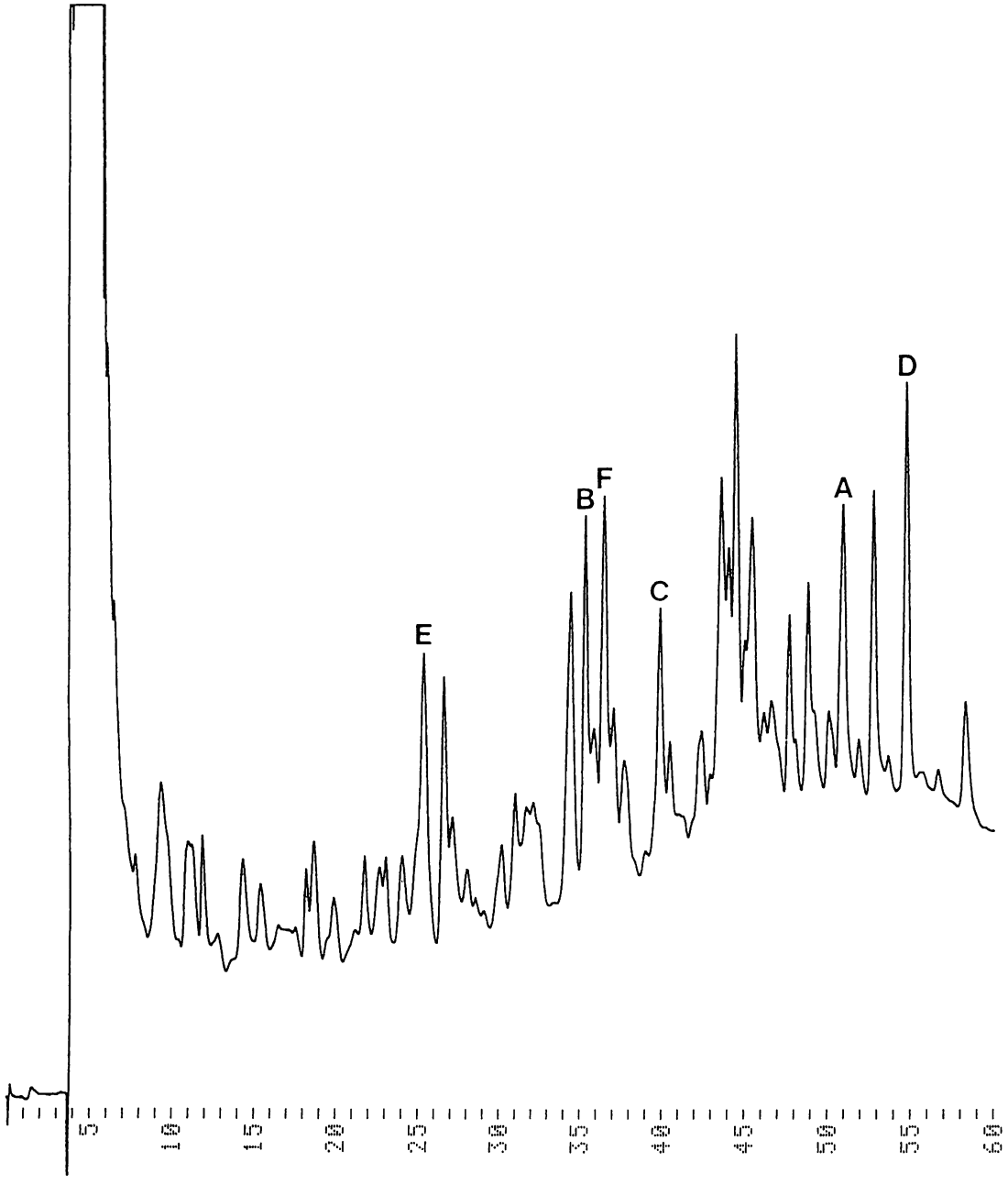

Fig.3 (a). Amino acid sequencing of the tryptic peptides of p80.
Fractionation of the tryptic peptides of p80. The p80 protein in (a) was eluted from the membrane and digested with TPCK-trypsin. The proteolytic fragments were then separated by HPLC. Vertical axis represents the relative absorbance at 215 nm. Peaks A and B were applied directly to an automated gas-phase microsequencer, and their sequences were determined. The samples of peaks C, D, E and F were applied to the microsequencer after rechromatography.

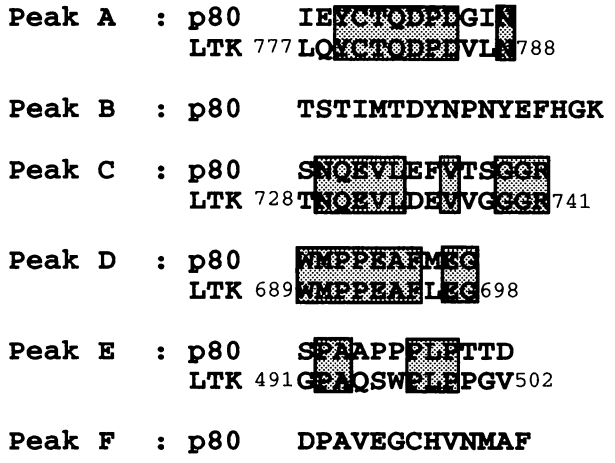

Fig.3. (b) Amino acid sequencing of the tryptic peptides of p80.
Amino acid sequences of the tryptic peptides. Homology research revealed that peaks A, C, D, and E had some homology with Ltk. Peaks B, and F had no homology with any registered proteins.

three cases that had been shown previously to carry t(2;5). The presence of t(2;5) in other cases were confirmed by the introduction of RT-PCR. Two primer sets covering this transgene at chromosome 2 site and 5 site were introduced so that the product can be derived only in cases that carry t(2;5). This RT-PCR system worked beautifully on control cases on which only cases with t(2;5), On ten Ki-1 lymphomas studied, three cases were found to produce PCR product at expected size. And these three cases were shown to be the very three cases that were previously shown to carry t(2;5). Now, when these 10 cases, together with 20 non-Hodgkin's lymphomas other than Ki-1 lymphoma were immunostained with anti-p80, only these with t(2;5) were immunostained, showing apparently that p80 is expressed in Ki-1 lymphomas with t(2;5) exclusively (4).

The presentation ended here and it was now open for discussion.

Dr. Nakamine asked whether the functional phosphotyrosine shown on kinase assay and p80 are the same molecule. Prof. Mori answered that the they are identical and that the protein was revealed to autophosphorylate.

Prof. AC Feller commented that he was fascinated hearing this research and asked whether Prof. S. Mori studied other lymphomas by this antibody. Prof. S Mori answered that he surdied over 20 cases of lymphomas other than Ki-1 lymphoma but could not find any positive cases.

Dr.Nakamine asked a possibility that p80 can be a tumor marker. Prof. S. Mori answered that p80 should be regarded as a specific marker for a certain subgroup of Ki-1 lymphoma that are related to t(2;5). (Actually, on later study of Shiota et al., Ki-1 lymphoma was

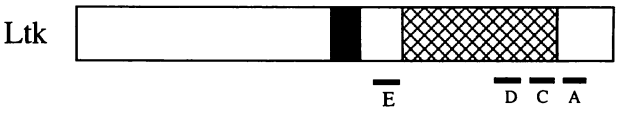

Fig.3. (c) Amino acid sequencing of the tryptic peptides of p80.
A schematic structure of Ltk and identification of sequences homologous to p80. Positions of sequenced peptides showing homology with Ltk are indicated by small bars at the bottom. The transmembrane region and the kinase domain are indicated by a black box and cross-hatche box, respectively.

separated into p80-positive and -negative subgroup. Dr. Shiota et al. showed various important differences, including age of onset and natural course, between these subgroups[5]).

Prof. Namba asked why only a part of Ki-1 lymphomas were positive for p80. Dr. Mori speculated that the incidence reflects the real incidence of t(2;5) among Ki-1 lymphomas.

Dr. Takeshita asked whether p80, so far could not demonstrate the constant association of CD30 molecule and p80, even though the immunocoprecipitation of anti-CD30 could sometimes demonstrate p80 beautifully. It was also his comment that there should definitely be the association in expression of these two molecules, probably not direct but somehow indirect way.

Key words: lymphoma, Ki-1, CD30, tyrosine kinase, p80, t (2;5), Ltk

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