Enhanced Viral Transformation of Skin Fibroblasts from Neurofibromatosis Patients*

PASCUAL BIDOT-LOPEZ, M.D. and JACK W. FRANKEL, Ph.D.†

Tampa Branch Laboratory, Department of Health and Rehabilitative Services, Tampa, FL 33601

ABSTRACT

Enhanced viral transformation of cultured skin fibroblasts (SF) from patients with neurofibromatosis (NF) was observed, compared with cultures established from normal, age-matched controls. Cultures of skin fibroblasts from persons with and without clinical NF in families in which the disorder had been diagnosed were examined for transformability by Kirsten murine sarcoma virus. The viral transformation results were compared with those obtained with SF cultures initiated from controls in families without history of any disorder with an hereditary component, or cancer. The data show that 63 percent of cultures from patients with clinical NF were transformed, compared with 7 percent of control cultures ($P = <0.0054$). Cultures of skin fibroblasts from persons without the classical features of NF, but in families in which the disorder had been recognized, also exhibited a relatively high transformation rate, since 75 percent were transformed. Neurofibromatosis can be included among other hereditary disorders in which enhanced transformability of cultures of SF by an oncogenic virus may be demonstrated.

Introduction

Neurofibromatosis (NF) is an autosomal dominant disorder with a population frequency of about 1 in 3,000.2 The presence of multiple café-au-lait spots, axillary freckles and cutaneous tumors (neurofibromas) are considered by many clinicians as pathognomic,14 although these criteria are not characteristic for this disorder only. Additional difficulties are encountered in the diagnosis of NF that relate to changes in penetrance and variability of expression within families. The tendency towards neoplasia may be considered a facet of NF in view of the frequent occurrence of neurofibrosarcomas, malignant schwannomas, and other less common tumors, such as...
Wilms’s tumor, rhabdomyosarcoma, and several types of leukemia.\textsuperscript{1,9,20} A reliable \textit{in vitro} marker for NF has not been established, nor can one predict those patients whose tumors will transform to malignancy, or who will develop new tumors. Comprehensive reviews of the current knowledge of the nature and pathogenesis of NF are presented elsewhere.\textsuperscript{13,15}

Cultured skin fibroblasts (SF) from patients with certain autosomal dominant precancerous states, e.g., adenomatosis of the colon and rectum\textsuperscript{11} and its variant, Gardner’s syndrome\textsuperscript{5} and conditions that predispose to leukemia, e.g., autosomal recessive Fanconi’s anemia syndrome\textsuperscript{4} and Down’s syndrome,\textsuperscript{8} exhibit increased sensitivity to oncogenic virus transformation.\textsuperscript{7,10,18,19}

The data presented in this report show the occurrence of enhanced viral transformation of cultures of SF established from patients with NF. The questions of degree of relevance of this finding in terms of presentation of a reliable genetic marker for NF and as a factor in the prognosis of neoplasia are yet to be answered.

### Materials and Methods

**Skin Biopsy Sources**

Male and female patients exhibiting the generally accepted clinical criteria for NF and family members without clinically-apparent disease were included in the study (table I). Control participants in the same sex proportions consisted of those whose history and physical examination did not reveal any familial disorders or cancer. Skin biopsies were collected from controls at the same time as subject specimens and handled in parallel, step by step, as those from patients with NF. All study participants were fully apprised of the skin biopsy technique and motives for this research, questions answered, and appropriate informed consent materials signed that had been approved by the Human Investigation Committee, University of South Florida Medical Cen-

---

**TABLE I**

Transformation by Kirsten Murine Sarcoma Virus (KiMSV) of Skin Fibroblast (SF) Cultures Established From First Degree Relatives in Families with Neurofibromatosis (NF)*

<table>
<thead>
<tr>
<th>Family</th>
<th>Code</th>
<th>NF</th>
<th>Age</th>
<th>Race</th>
<th>Relation</th>
<th>Café-au</th>
<th>Cutaneous</th>
<th>Axillary</th>
<th>KiMSV Endpoint†</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>ZCF</td>
<td>+</td>
<td>24</td>
<td>Hispanic</td>
<td>Sister</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>I</td>
<td>TCF</td>
<td>-</td>
<td>33</td>
<td>Hispanic</td>
<td>Sister</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>II</td>
<td>BCU</td>
<td>+ 1 1/2</td>
<td>Black</td>
<td>Son</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>&lt; 1</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>GCU</td>
<td>+</td>
<td>30</td>
<td>Black</td>
<td>Mother</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>DCU</td>
<td>-</td>
<td>24</td>
<td>Black</td>
<td>Aunt</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>II</td>
<td>OCU</td>
<td>-</td>
<td>25</td>
<td>Black</td>
<td>Uncle</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>III</td>
<td>MU</td>
<td>-</td>
<td>42</td>
<td>Black</td>
<td>Aunt</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>III</td>
<td>MJ</td>
<td>+ 30</td>
<td>White</td>
<td>Daughter</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>JJ</td>
<td>+ 55</td>
<td>White</td>
<td>Mother</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>DF</td>
<td>+</td>
<td>12</td>
<td>White</td>
<td>Female</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>V</td>
<td>MF</td>
<td>+</td>
<td>40</td>
<td>White</td>
<td>Female</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>VI</td>
<td>GT</td>
<td>+</td>
<td>71</td>
<td>Hispanic</td>
<td>Male</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

*Among normal, age-matched controls, 1 of 29 SF cultures was positive for KiMSV transformation (endpoint 1)

†Reciprocal Log\textsubscript{10} (positive for transformation when highest KiMSV transforming dilution is one or higher).

§Patient with skeletal abnormality

\&Patient with cystic hygroma

\#Patient with hemangioma

\$Patient with adenocarcinoma of the lung
ter. From the time of biopsy, patient confidentiality was assured through assignment of codes. The overall information relative to each patient was stored under lock by statisticians and was not made available for correlation until all laboratory assays had been completed.

Skin cells were obtained by punch biopsy (maximum 4 mm) of normal appearing flat skin in a cosmetically-unimportant area (scapula). The tissue samples were collected aseptically in cold Leibovitz L-15 medium containing 2 percent heated fetal bovine serum (FBS), gentamycin (100 μg per ml) and fungizone (50 μg per ml). This self-buffering medium provided a stable pH during transport of the tissue from surgery to the cell culture laboratory.

**SF Cultures**

The skin biopsy was transferred into a cell culture dish, the collecting fluid removed, and the tissue minced into small pieces with a sharp surgical scalpel. The tissue pieces (about four to five) were placed into each of two plastic petri dishes (60 mm) and incubated at 37° for one hour. Three ml of growth medium (GM) consisting of Eagle’s minimal essential medium in Earle’s balanced salt solution (MEM), supplemented with 2 mM glutamine, 20 percent FBS and penicillin (100 μ per ml), streptomycin (100 mg per ml), and fungizone (25 μg per ml), were carefully added in order not to dislodge the adhering tissue bits. The culture dishes were incubated at 37° in a five percent CO₂ air atmosphere with medium changed every three to four days. After one to three weeks, depending on time to monolayer development, the cells were removed with an EDTA-trypsin mixture, pooled, washed, transferred to two culture dishes (120 mm) containing five ml of GM and incubated at 37° (passage 2). When confluent (the cells of passage 4), the monolayers were used for viral transformation assays. All cell suspensions were monitored to exclude mycoplasma and other microbial agents, and the cells were stored in liquid nitrogen as a resource.

**Kirsten Murine Sarcoma Virus (KiMSV)**

KiMSV was prepared in a normal rat kidney cell line (NRK) transformed in vitro by KiMSV, fluids collected 48 hours after plating, clarified by centrifugation, passed through a 45 μ Millipore filter and stored in liquid nitrogen. A single standard pool of KiMSV was used in all viral transformation assays.

**Viral Transformation Assay**

The morphological alterations of the KiMSV transformed foci are characterized by refractile spindle-shaped and round cells which grow on top of the monolayers and exhibit large cytoplasmic vacuoles. Viral transformation assays were conducted 24 hours after 1 × 10⁶ SF cells harvested from passage 3 were inoculated into petri dishes. The cultures were treated for one hour at 37° with DEAE-dextran (MW > 2 × 10⁶ daltons; final concentration 25 μg/ml for 30 minutes in a total incubation volume of 4 ml) and incubated with 0.25 ml of serial 10-fold dilutions (10⁻¹ to 10⁻⁹) of the standard frozen KiMSV stock. Two cultures, each inoculated with one of the virus dilutions, were maintained at 37° and the medium replenished as necessary. The tests were observed 14 days after KiMSV inoculation and results recorded as the reciprocal of the highest KiMSV dilution that induced transformation of SF (i.e., one or more areas of characteristic cytologic change) in at least one of the duplicate culture flasks. The criterion for distribution of cultures of SF into the positive transformation category was whether the transforming KiMSV
dilution was one or greater. As controls during each transformation assay, the infectious titer (TCID\textsubscript{50} per ml) of the KiMSV standard stock was assayed for conformity in NRK cells. Transformation dilution endpoints of KiMSV were also determined in a positive control of culture of SF established from a donor with Gardner’s syndrome and a negative control culture from a normal individual without history of any hereditary disorder or cancer.

**Statistical Analysis**

Owing to the small sample size, the 2 x 2 contingency table was used to analyze the statistical significance of the data.\textsuperscript{17}

**Results**

A majority of cultures of SF established from patients with clinically-diagnosed NF in families in which the disorder had been previously described, as well as cultures initiated from asymptomatic first degree relatives, were transformed by KiMSV and compared to normal, age-matched controls. In families affected with NF, there was no significant differences in percent of cultures of SF transformed whether or not the skin biopsy donor showed clinical evidence of NF. The data presented in table 1 show that 63 percent of cultures of SF from symptomatic patients with NF were transformed, as were 7 percent of control cultures (P = <0.0054). Cultures of SF from totally asymptomatic first degree relatives in families in which the disorder had been described exhibited a high degree of transformability in that 75 percent were transformed, compared to 7 percent of controls (P = <0.0183).

The KiMSV transforming dilutions summarized in table I were obtained through performance of two separate tests. Each of the assays was controlled by use of NRK cells, SF from a Gardner’s syndrome patient, and a normal individual. In NRK cells, comparable results were obtained in terms of viral infectivity (range 10\textsuperscript{6.6} and 10\textsuperscript{4.9} TCID\textsubscript{50} per ml) and transformability (positive and negative controls, 3 and <1, respectively).

In other studies, cultures of SF established from patients DCU, DF, GT, and four control individuals were retested for transformability utilizing KiMSV, and none were transformed. No quantitative differences were observed in cell cultures derived from skin biopsies obtained from the café-au-lait spots and normal appearing skin from the same individual. Although all data reported in table I were obtained with SF cultures at the fourth cell passage level, the viral transformation results were statistically comparable to tests performed at other passage levels (i.e., 2, 6, and 9) and with cells that had been frozen for over three years in liquid nitrogen.

**Discussion**

The study reported here indicates that NF may be added to the list of hereditary disorders in which cultured SF exhibit enhanced transformability by an oncogenic virus. Skin fibroblasts from persons not showing the characteristic clinical picture of NF, but in families in which the disease had been described, also were transformed by KiMSV.

Since the clinical description of NF may conflict with that of other disorders, it is possible that an incorrect diagnosis could have been established for some individuals who appeared to be specifically affected with NF. This could account for the absence of viral transformation in cultures of SF initiated from an individual with clinically-diagnosed NF (DF, table I) who was a first degree relative in a family in which NF had never been reported. Neurofibromatosis may represent a spectrum of diseases clinically resembling one another, although subtle differ-
ences in genetic disposition may prevent detection of the gene required for transformation. The gene for viral transformation may reside in different chromosomes and/or in the same chromosome, although distant, and together with the gene for NF may be indicative of an epigenetic phenomenon.

The response (73 percent) of cultures of SF considering all patients affected with NF that were transformed by KiMSV was comparable to the value (71 percent) obtained in recent studies conducted with cultures of SF established from patients with lung cancer. In view of the similarity in relative numbers of cultures of SF that were transformed in these two investigations, the viral transformation data could reflect a factor(s) in addition to the gene(s) in question. Among cultures of SF established from control individuals, the percent of cultures transformed in the NF and lung cancer studies was 7 percent and 4 percent, respectively. There are other reports that present evidence for KiMSV transformation of cultured SF from apparently normal human cell lines. In this context, it will be of relevance to determine whether or not cultured SF from persons in families not affected with NF, but with cutaneous neurofibromas or neurofibrosarcomas, also show evidence of transformation. The significance of enhanced KiMSV transformation of cultures of SF from apparently normal donors is difficult to understand. A heretoforeundiagnosed hereditary disorder could be present, and not as yet expressed, or these persons may be at risk for development of neoplasia.

Although the results show enhanced viral transformation of cultures of SF initiated from individuals affected with NF, much work is yet to be done before this procedure may be considered as a diagnostic tool for NF. Other studies reveal differences in growth characteristics of cultured SF from patients with NF. These SF are resistant to inhibition by 3-nitrotyrosine and 6-hydroxydopamine, grow more slowly, stop growing at a lower population density, and are larger, pleomorphic, and fail to form confluent monolayers when growth ceases, when compared with normal cells. The observation of enhanced viral transformation of SF from NF patients is clearly not specific, since a similar phenomenon had also been found in a variety of other conditions. Our studies were concerned only with NF patients exhibiting the classical clinical signs of the disorder and their near relatives. Transformation studies have not as yet been performed with large numbers of patients suspected of having the gene for NF, but in whom accurate diagnosis presents a problem. There is no need for an NF diagnostic test for patients in whom the disorder may be diagnosed clinically, although the possible utility as a monitoring technique in genetic counseling should not be overlooked. Transformation of cultures of SF with relatively low doses of KiMSV may be indicative of subsequent development of malignant tumors. In this context, the viral transformation test could prove useful, provided the procedure could predict those patients who are genetically predisposed to develop these tumors.

References