Detection of Malignant Cells in Cerebrospinal Fluid Using Fluorescence In Situ Hybridization

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Abstract. Cytologic examination of cerebrospinal fluid (CSF) is the diagnostic gold standard for leptomeningeal metastasis (LMM). However, this technique is only moderately sensitive when routine staining procedures are applied. The use of fluorescence in situ hybridization (FISH) to identify malignant cells may have an additional value in diagnosing LMM, since numerical chromosomal aberrations (NCA) can be detected at the single cell level. We tested the feasibility of FISH to detect tumor cells in CSF by analyzing 22 samples with proven LMM with a probe for chromosome 1 (1q12) to detect NCA in the cells. A control group consisted of samples from 10 patients with inflammatory neurologic disease. In 7 LMM samples no cells or only lysed cells were found, due to time delay before fixation. Of the other 15 LMM samples analyzed, 13 showed NCA (87%), while no NCA were found in the control group.

Our results indicate that FISH may be a useful additional diagnostic tool to the cytodiagnosis of CSF in cases of LMM. We expect that FISH can become an additional marker for malignancy at the single cell level in patients with LMM, which may also be of use to determine the effect of therapy for LMM.

Key Words: Cerebrospinal fluid; Diagnostic cytology; Fluorescence in situ hybridization; Leptomeningeal metastasis.

INTRODUCTION

Leptomeningeal metastasis (LMM) is an important and often disabling neuro-ological complication of cancer (1, 2). The total incidence of LMM is unknown, but autopsy data have shown that it occurs in approximately 8% of systemic cancer (3). LMM develops in 7 to 15% of patients with non-Hodgkin lymphoma and in 5 to 15% of patients with leukemia. Adenocarcinomas are the most common solid tumors to metastasize to the leptomeninges. For example, breast cancer accounts for 22 to 64% of LMM in patients with solid tumors, followed by lung cancer (1 to 26%) and malignant melanoma (7 to 15%) (4, 5).

Early identification and treatment of this complication of systemic cancer may prevent serious neurological symptoms (6). When LMM is suspected, it can be confirmed by the identification of malignant cells in the cerebrospinal fluid (CSF) (1, 2, 6, 7), but even after repeated cytological examinations, 15 to 20% of the suspected cases remain negative when conventional staining procedures are applied in the cytodiagnostic approach (1, 7, 8). The reasons for this large number of false-negative cases include the morphological resemblance between malignant cells and ependymal cells, the non-malignant aspect of lymphocytes in leptomeningeal localization of non-Hodgkin lymphoma or leukemia, the presence of blood cells due to traumatic lumbar puncture, and the fact that often only a small number of cells can be evaluated (9, 10). Because a negative cytological examination does not exclude LMM, additional diagnostic tests have been proposed, including biochemical marker assays (11), immunocytochemistry (12, 13), flow cytometric detection of aneuploidy (14), the polymerase chain reaction in cases of LMM of leukemia with known genetic alterations (15), and neuro-imaging using magnetic resonance (16, 17). Although these diagnostics can support the diagnosis, the sensitivity of these tests did not turn out to be high enough to improve the diagnosis of LMM (2, 5).

Since genomic aberrations often parallel malignancy, the in situ hybridization (ISH) technique, by which chromosomal changes can be detected in interphase nuclei by using specific DNA probes, has been suggested as a possible additional diagnostic aid. Due to recent developments such as the generation of different types of probes and the development of different pretreatment steps, this technique can indeed become an important adjunct in the diagnosis of malignancy (18–21). This procedure has so far been applied to cytological specimens of fine needle aspirates of various neoplasms (22), bone marrow aspirates (23), and bladder washes (24) to detect NCA, but until now it was not used in the cytodiagnosis of CSF. We tested the feasibility and usefulness of ISH in CSF of 22 patients with cytologically proven LMM of systemic cancer and in 10 controls with non-malignant disorders. A centromeric DNA probe for chromosome 1 (1q12) was applied to detect aneuploid cells because of the high percentage of breast carcinoma cases in our series and the finding that in this type of cancer numerical aberrations for chromosome 1 can be detected in more than 80% of the cases (25–27).
MATERIALS AND METHODS

Patient Material

CSF samples were obtained from 22 patients with LMM of systemic cancer (12 breast carcinomas, 1 melanoma, 4 non-Hodgkin lymphomas, 2 leukemias, 1 ependymoma, 1 small cell lung carcinoma and 1 unknown primary tumor) by lumbar puncture, using a 20-gauge syringe. All samples were Giemsa stained and judged to be malignant by 2 experienced pathologists (PvH, JWA). A disease control group consisted of 10 patients with proven bacterial (n = 7) or viral (n = 3) meningitis.

In Situ Hybridization

The aliquots from the CSF of the patients with LMM and the control group were either treated with pepsin prior to cytosin preparation or after cytocentrifugation. Pretreatment of the cytological specimen was done as described earlier (28, 29). Cells were treated for 20 minutes at 37°C in 100 μg pepsin (from Porcine stomach, 2500 to 3500 units per mg protein; Sigma, St. Louis, USA) per ml 0.01 N HCl to make the cells more accessible to the DNA probe. Following the pepsin digestion step, the slides were air dried at room temperature and thereafter dehydrated in an acidiﬁed ethanol series. After dehydritization, the cells were ﬁxed in 1% formaldehyde in phosphate-buffered saline (PBS) for 15 minutes at 4°C. After ﬁxation, the coated glass slides were washed twice in PBS and subsequently dehydrated in 70%, 90% and 100% ethanol.

In situ hybridization was performed essentially as described by Hopman et al. (28). The centromeric DNA-probe for chromosome 1 (t(14,2), pUC 1.77; Cooke and Hindley [30]) was hybridized in single-targetISH in a hybridization buffer containing 2× SSC (0.15 NaCl, 15 mM Na-citrate pH 7.0), 60% formamide, 10% dextran sulphate, 0.2 mg/ml yeast RNA, and 0.2 mg/ml herring sperm DNA under a coverslip, and denatured at 70°C for 5 minutes. Thereafter, the slides were placed in a moist chamber and incubated overnight at 37°C. The slides were then washed 2 times in 2× SSC/0.05% Tween at 45°C, once in 0.1× SSC at 60°C, and ﬁnally once in 4× SSC/Tween 0.05% at room temperature. The biotinylated chromosome 1 probe was detected with ﬂuorescein-isothiocyanate (FITC) conjugated to avidin (Vector Laboratories, Burlingame, USA). If necessary, the signal was intensiﬁed by a subsequent incubation with biotinylated-goat-anti-avidin (Vector Lab.) and avidin-FITC (Vector Lab.). Nuclear counterstaining was done with 4,6-diamino-2-phenylindole (DAPI, Sigma, St. Louis, MO, USA).

Slide Evaluation

Because of the low number of cells that could be analyzed in the CSF samples, no cut-off level for abnormality could be assessed. The following criteria were applied to assess the validity of specific chromosomal aberrations (28): (a) overlapping nuclei are not included in the evaluation; (b) nuclei should have more or less the same FISH signal intensity; (c) the nucleus is DAPI-positive; (d) the nucleus has a low autofluorescence background; (e) nuclear morphology should be acceptable.

Samples were classiﬁed as disomic if no cells with more than 2 FISH signals were observed. If cells with more than 2 FISH signals were observed, they were regarded as aberrant if the above criteria applied, and were then classiﬁed as trisomic, tetrasomic or polysomic. Since in the case of 4 signals per cell one might argue that cells with this chromosome content represent the G2/M phase of the cell cycle, we refer to Beck et al. (31), who have proven that normal G2/M cells exhibit only 2 signals per chromosome.

Fluorescence Detection and Photography

Fluorescence microscopy was performed using a Leica DMBRE microscope equipped with the appropriate epifluorescence filter set for FITC and DAPI. Images were captured and stored with the Metasystem Image Pro System (Heidelberg, Germany), including a black and white charge-coupled device (CCD) camera.

RESULTS

Control Samples

The 10 control samples contained large numbers of cells that could be analyzed (at least 100 nuclei per sample). These cases, diagnosed as infectious neurological disease, showed no NCA, in that cells with more than 2 FISH signals were not detected. Figure 1 shows an example of the FISH results on cells in the CSF of a patient with a bacterial meningitis, revealing mainly disomic cells.

Although no malignant cells were found in these samples, occasionally a few cells showed cytomorphologically atypical features, which were, however, not interpreted as abnormal because of the large number of normal white blood cells. The percentage of cells showing one FISH signal ranged from 2 to 17%, which is to be expected on the basis of colocalization of chromosome targets or interpretation problems. Overall, the cytomorphology of these benign samples was better than that of the malignant ones. The FISH signals were of good quality, with minimal differences in fluorescence intensity. Occasionally, cells with minor binding sites were observed.

Malignant Samples

The cytological parameters that established the diagnosis of LMM, as well as the FISH results and the relevant morphological aspects of the cells evaluated by FISH, are summarized in Table I. Cytological diagnosis of malignancy was not only based on nuclear aspects such as hyperchromatism or anisokaryosis, but also on features such as an increased nuclear-cytoplasm ratio, increased cell number and the presence of clusters of cells. Only 15 of the 22 cases could be analyzed by FISH because of the absence of cells in 2 cases and the presence of lysed cells in 5 cases. Cell lysis was judged to be present when the nucleus had lost its integrity or its morphology. Lysis of cells was present mostly in those samples subjected to a longer time delay (up to 24 hours) before ﬁxation. Thirteen of the 15 evaluable malignant samples (87%) showed varying numbers of cells with
increased FISH signal numbers, ranging from 3 to 7 signals per cell.

Figures 2 to 4 show several examples of FISH results obtained with the chromosome 1 probe in cytopins of CSF samples of patients with LMM. Figure 2 shows a trisomic cell surrounded by disomic cells in a case of LMM of breast cancer. Figure 3 shows intact nuclei with 2 and 3 FISH signals in a case of LMM of breast cancer. These cells are surrounded by lysed cells in which no conclusive FISH signal could be observed. Figure 4, finally, illustrates the FISH results in cells from the CSF of a patient with LMM of breast cancer. Cells with 5 and 7 FISH signals can be seen and a cluster of cells is also present.

In 2 cases (nr. 18 and 29), only cells with 2 FISH signals for chromosome 1 were observed. To test the feasibility of a repeated FISH analysis on the same slide, incubation with a centromeric DNA probe for chromosome 15 was
<table>
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<th>Case #</th>
<th>Primary diagnosis</th>
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</table>

Abbreviations: FISH, Fluorescence in situ hybridization; NE, Not evaluable; CSF, cerebrospinal fluid; SCLC, small cell lung cancer; ALL, acute lymphoblastic leukemia; NHL, non-Hodgkin lymphoma. Cytodiagnostic criteria (no. 1–9): (1) Increased cell number, i.e. more than 10 cells per μl; (2) Anisokaryosis; (3) Hyperchromatism; (4) Polymorphism; (5) Nucleolus present; (6) Increased nuclear-cytoplasmic ratio; (7) Mitotic figures; (8) Atypical lymphoid cells; (9) Clusters of cells.

Performated after removal of the hybridized probe for chromosome 1 in one of these cases (nr. 18). The same hybridization protocol was used to detect NCA for chromosome 15. In this case we could demonstrate a trisomy for chromosome 15.

Pitfalls

Several methodological problems and pitfalls became apparent during our study. One of the most serious problems that was met was loss of good cytomorphic, mainly due to time delay before fixation of the CSF-samples. Because of cell lysis, the difference in fluorescence intensity was relatively high and the discreteness of the FISH signals was lost.

DISCUSSION

Suspected LMM of systemic cancer is usually confirmed by the identification of malignant cells in the CSF (1, 2, 4, 7). Since a negative cytodiagnostic result does not exclude LMM, we undertook a study to test the feasibility and diagnostic value of the FISH technique to increase the sensitivity of CSF examination. The FISH method has been applied to several solid tumor types (18–20, 32, 33), and it has also been used to detect neoplastic cells present in cytological specimens such as fine needle aspirates (22), bone marrow aspirates (23), and bladder washes (24). CSF is almost ideally suited for evaluation by FISH because the cells present are already in suspension, so that minimal sample preparation is required.

In this study, we selected the chromosome 1 probe (1q12) to detect aneuploid cells of LMM in CSF, mainly because our series contained a high number of cases of LMM of breast cancer, in which chromosome 1 aberrations are frequently observed (25–27). We were able to demonstrate NCA in 87% of the evaluable cases with cytologically proven malignant cells, while none of the benign samples could be classified as abnormal (aneusomic) on the basis of the FISH procedure. The finding of 2 to 17% cells with one FISH signal in this control group is not a sign of abnormality, but a result of colocalization of chromosomal targets or problems in the macroscopic evaluation of the FISH signals. It seems reasonable, therefore, to expect that the FISH technique can
be informative in CSF cases where no malignancy can be demonstrated after routine cytodiagnosis.

The number of cases in which aberrations are detected might be increased by using more than just one probe, either in a repeated FISH procedure or by simultaneous hybridization with differently labeled probes. For this reason we performed a repeated FISH incubation in one case, using the same slide but with a different centromeric DNA probe, and were able to show NCA in this way. Furthermore, although a relatively large number of NCA were found in lymphomas, detection of specific translocations may increase the sensitivity of the FISH procedure applied to cells in CSF in cases of LMM of lymphomas. It may be obvious that the choice of the DNA probes depends strongly on the type of malignancy. If samples of the primary tumor are available, these can be used to screen an extended set of probes for selection of a specific marker to be applied to CSF cells.

When comparing the results of routine cytodiagnostic procedures with the FISH classification, the following 2 aspects should be kept in mind. The diagnostic results obtained by routine cytopathology are not only based on nuclear aspects, but also on other criteria, such as the presence of clusters of cells, increased cell numbers, and a high nuclear-cytoplasmic ratio (10, 34). These features cannot be evaluated with the FISH procedure. Therefore, we propose to perform a FISH analysis on the Giemsa-stained slide that was used for routine cytodiagnosis in order to detect NCA as a sign of malignancy whenever the cytodiagnostic evaluation reports atypical cells suspect for malignancy. Secondly, in our experience, interpretable FISH results are only obtained if there is proper cell morphology. Dux et al (35) showed that within 90 minutes after lumbar puncture, 90% of the cells were lysed when CSF was kept at room temperature.

Studies on the diagnostic utility of aneuploidy detection in CSF have been performed before. Cibas et al (14) detected DNA abnormalities in 69% of cases by using flow cytometry (FCM). The FISH technique has several advantages for detecting DNA abnormalities over FCM. Firstly, FCM requires a larger number of cells, whereas FISH can be performed at the single cell level. Secondly, tetrasomic cell lines are not easily recognized with FCM or mistaken for G2M cells, whereas FISH does detect these cells. One might argue that in the case of 4 chromosome signals per cell, these cells represent the G2M phase of normal cells. Beck et al (31) have, however, proven that normal G2M cells exhibit 2 signals when using the 1q12 probe and that no evident duplication of spot numbers occurs during DNA replication.

In conclusion, this study demonstrates the feasibility of using FISH on cells from CSF to detect NCA for the identification of malignancy. This technique may have additional value in the cytopathological evaluation of CSF, as has already been demonstrated for other cytological preparations. FISH is particularly suited for CSF analysis because of the often small number of malignant cells in the preparations.

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Received December 27, 1996
Revision received March 17, 1997
Accepted March 18, 1997