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Assessment of percutaneous fine needle aspiration cytology as a technique to provide diagnostic and prognostic information in neuroblastoma

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Abstract

The International Neuroblastoma Staging System (INSS) criteria for diagnosis requires an unequivocal pathological diagnosis and favours the identification of prognostic markers in the samples. Surgical biopsies of the primary tumour and bone marrow (BM) sampling in metastatic disease constitute the major sources of tumour material for the laboratory. We analysed the possibility of percutaneous fine needle aspiration cytology (FNAC) constituting an alternative procedure to the conventional technique of sampling of the primary tumour in children with advanced neuroblastoma. From July 1987 through July 1998, 64 consecutive children suspected of having advanced neuroblastoma and referred to our institution underwent percutaneous FNAC of deeply located tumours. FNAC was performed using 22-gauge needles under ultrasound guidance, before any chemotherapy and within the first days following admission. No complication occurred after FNAC. The median number of the extracted tumour cells was 2.3×10^6 (range: 0–40.6×10^6). Cytology analysis was possible in 59/64 cases (92%) and immunocytochemistry in 56/64 (88%) allowing confirmation of the diagnosis. *N-Myc* analysis was available in 46/64 (72%). In addition, the presence of a partial deletion of chromosome 1p (del 1p) was assessed, since 1992, in 24/47 cases (51%), where enough cells were available. FNAC of deeply located advanced neuroblastoma is safe and information is available in a few hours after admission. The provided material is reliable for confirmation of diagnosis and analysis of biological prognostic markers in the majority of cases. More invasive tumour sampling procedures are required only in selected cases.

Keywords: Childhood cancer; Neuroblastoma; Percutaneous biopsy; Cytology; Immunocytochemistry; Cytogenetics

1. Introduction

The International Neuroblastoma Staging System (INSS) requires cytopathological or histopathological confirmation of the disease [1]. Currently, most protocols recommend primary radical resection only for localised tumours. In these cases, material for diagnosis and biological studies is available, allowing histopathological classification according to the Shimada system [2].

In the more frequent cases, such as advanced localised neuroblastomas or metastatic neuroblastoma (INSS stages 4 and 4S), up-front surgical resection is not indicated, but tumour material is needed for diagnosis and biological assessment. Surgical biopsies or percutaneous core biopsies are carried out to provide histological diagnosis and prognostic information. However, the Shimada histopathological classification system developed for distinguishing favourable and unfavourable groups requires a large tumour sample. Surgical or core biopsies only provide a small amount of tumour which constitutes a limitation of such procedures, especially in Shimada's nodular histological subtype.

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Biological characteristics of the tumour such as *N-Myc* oncogene amplification [3–6] and loss of chromosome 1p [7,8] provide major information of prognostic relevance. More recently, other biological features such as the expression of cluster differentiation (CD) 44 [9,10] and of the receptor of the nerve growth factor tyrosine receptor kinase-A (TRK-A) [11,12] have also been described as of prognostic value. Nevertheless, it is uncertain to what extent complete histological and biological evaluation will alter the outcome of treatment of neuroblastoma and hence the need for prospective documentation. There are also several concerns regarding the systematic practice of surgical biopsies (morbidity rate, cost and delay time before surgery). We therefore conducted this study to evaluate the safety and the reliability of pretreatment fine needle aspiration cytology (FNAC) of deeply sited neuroblastoma as an alternative diagnostic tool.

2. Patients and methods

This single centre prospective non-randomised study was conducted at our institution from July 1987 through July 1998. All children with a presumptive diagnosis of neuroblastoma were admitted to the Department of Paediatric Oncology.

2.1. Patient eligibility

To be eligible in the study, patients were required to: (1) be under 15 years of age; (2) have a deeply located tumour suspected to be a neuroblastoma following clinical examination and catecholamine metabolite measurement; (3) have demonstrated radiographic evidence (on skeletal radiography or abdominal ultrasonography (US) or meta-iodo-benzylguanidine (m-IBG) scintigraphy) of neuroblastoma considered not totally resectable before chemotherapy. Neuroblastoma that were not totally resectable at onset were defined as metastatic tumours (INSS stages 4 and 4S) and tumours whose total removal would lead to the sacrifice of an adjacent organ or vessel damage. For locally advanced neuroblastoma, the amount of tumour that could be resected was assessed in conjunction with the paediatric surgeon in charge of the child, after a complete radiological work-up including US and computerised tomography (CT scan) or magnetic resonance imaging (MRI). Patients with dumbbell neuroblastoma were eligible for the study.

2.2. Patient ineligibility

Patients were ineligible for this study: (1) when presenting with suspected totally resectable neuroblastoma on clinical and radiological examination; (2) if tumour sampling was possible on peripheral tumour sites (skin nodules, superficial metastatic lymph nodes), bone metastases and serous fluid collections; (3) after the onset of any chemotherapy; and (4) in the case of presumptive recurrent tumours.

2.3. Radiological work-up

The extent of the disease was assessed before this study by a complete radiological work-up including plain radiography, US, CT scan or MRI. All children were evaluated for m-IBG scintigraphy.

2.4. Technique of puncture

The site to be sampled was selected on the basis of high contrast enhancement foci on CT scan or MR images. Initially, FNAC was performed in a radiology room under neuroleptanalgesia (atropine sulphate (0.01 mg/kg) + diazepam (0.5 mg/kg) + ketamine (2 mg/kg intravenously (i.v.)). Since August 1991, FNAC was performed in an operating theatre and combined with the insertion of a central venous catheter and bone marrow staging while under general anaesthesia. Normal coagulation parameters were required. The procedure was achieved in aseptic conditions. Standard spinal needles (Yale® spinal Becton Dickinson & Co, Grenoble, France) of 22-gauge 3.5 in (0.7×90 mm) were used for tumour aspiration. The ultrasound guidance (Hitachi ultrasound EUB 415, Hitachi® Medical Corp., Tokyo, Japan) enabled the sampling of any necrotic tumour to be avoided as well as any damage to vessels or vital organs to be minimised. The FNAC were performed 'free hand' by a radiologist and under real-time US so that the needle route was continuously controlled. Vacuum aspiration was obtained in 20 ml single-use syringes (Omnifix® B. Braun Melsungen AG, Melsungen, Germany) with the Cameco® system (Cameco® London, UK), by moving the needle slightly back and forth and turning it around its axis, as previously described [13]. An average of six needle passes were routinely performed in order to obtain sufficient material for analysis. The aspiration of any haemorrhagic material was avoided, as red blood cells interfere with immunocytological analysis and the removal of red blood cells from the sample usually leads to a waste of tumour cells. The needle passes fanned out from a single percutaneous access to different portions of the tumour. The radiologist achieved air-dried smears from the first samples and diluted the other samples in Roswell Park Memorial Institute (RPMI) 1640 solution. The tumour samples were then brought to the pathologist by the radiologist while bone marrow (BM) aspirates and trephines were performed and central venous access inserted. Additional FNAC could be performed if preliminary laboratory assessment revealed insufficient material.

2.5. Evaluation of complication rate

Before the patient left the operating room, US examination was performed to detect any bleeding. Every child had an in-patient clinical follow-up for at least 24 h, looking for acute complications. In the long-term, any recurrence on the track of the needle was documented.

2.6. Examination of the tumour material

The pathologist and the biologist were at hand to assess the quality and the quantity of the material selected for subsequent analytic techniques. The material included air-dried smears and fresh cells suspensions (diluted in RPMI 1640 solution).

2.7. Cytological analysis

Air-dried smears were studied after Wright colouration. Immediate examination by the pathologist allowed a rapid assessment of the presence of malignant cells. The sample cellularity was roughly evaluated by a rapid morphological assessment of a cytospin after Wright colouration and by counting the cells on a Malassez slide. When samples were inadequate, repeat punctures could immediately be performed.

2.8. Immunocytochemistry

Six to twelve centrifuged smears were immediately prepared for immunocytochemistry staining (1×10^5 cells per smear). Analysis of markers anti-CD45 and anti-CD56 [14] by double immunofluorescence [15] using anti-CD45 conjugated with fluorescein isothiocyanate (FITC) (Becton Dickinson, Pont de Claix, France) and anti-CD56 conjugated with phycoerythrin (PE) (Becton Dickinson) enabled us to calculate precisely the percentage of malignant cells in a period time of 45–60 min after the FNAC puncture. Additional FNAC sampling could then immediately be performed if required. When the cytological analysis demonstrated the presence of neuropil and differentiating neuroblast cells with ganglionic maturation, these markers were considered sufficient to confirm the diagnosis of neuroblastoma. In cases of undifferentiated cells on the cytological analysis, the pathologist performed additional markers during the next 24 h to exclude lymphoproliferative diseases, primitive neuroectodermal tumour (PNET) or rhabdomyosarcomas. The markers included anti-CD3, anti-CD20, anti-CD99 and anti-desmin by an avidin–biotin complex technique and alkaline phosphatase method. The markers were also performed in case of normal catecholamine levels or normal m-IBG scan. The analysis by the biologist of TRK-A [11,12] and CD44 [9] were performed later to evaluate tumour prognosis.

2.9. Molecular biological analysis

N-Myc amplification analysis by Southern blot required $1.5–2\times10^6$ cells with the percentage of tumour cells exceeding 50%. The minimum number of tumour cells required was 7.5×10^5 to 10^6. Cells were centrifuged and frozen at $-70°C$. The analysis of the ploidy and deletion on chromosome 1p (del 1p) analysis on the extracted cells were not parameters included in the prospective study in 1987. However, we recorded in this series the results of del 1p analysis since 1992. This analysis was performed by fluorescence *in situ* hybridisation (FISH) [16].

3. Results

3.1. Patient characteristics

204 patients with neuroblastoma were admitted to our institution during this 11-year period. 64 patients (36 (56%) male/28 (44%) female) were eligible for this study. Mean age was 32 months (range: 5–168). The distribution was: advanced localised stage: $n=20$ (31%), INSS stage 4: $n=37$ (58%) and INSS stage 4S: $n=7$ (11%). Tumour location was retroperitoneum ($n=58$; 91%), posterior mediastinum ($n=2$; 3%), pelvis ($n=2$; 3%) and liver ($n=2$; 3%). One child amongst the 2 cases in posterior mediastinum had a dumbbell neuroblastoma in whom surgery was not indicated. The 2 cases with FNAC on lesions of the liver were 1 child presenting a stage 4 neuroblastoma with large cystic adrenal mass and another child presenting a stage 4S neuroblastoma with a primary tumour of limited size. Excluding liver metastases, the tumour sizes ranged from 12 to 2912 cm^3 (mean = 600 cm^3).

3.2. Technical results

FNAC was achieved within 48 h following admission to our hospital in the majority of cases and the delay time never exceeded 3 days. The duration of the procedure was in most cases less than 20 min. In less than 20% of patients, additional FNAC samples were performed to increase the amount of extracted cells before the end of general anaesthesia was decided.

3.3. Complication rate

We did not observe any immediate or delayed complication related to the procedure. In particular, no child experienced intra-abdominal or retroperitoneal bleeding, haematuria or bowel perforation following FNAC. No recurrence developed within the needle track.

Table 1
Number and percentage of patients according to the number (n) of tumour cells extracted

Number of tumour cells (n)	Patients n (%)
$n < 200\,000$	10 (16)
$200\,000 \leqslant n < 1.2\times10^6$	12 (19)
$1.2\times10^6 \leqslant n < 4.2\times10^6$	22 (34)
$4.2\times10^6 \leqslant n < 7.4\times10^6$	8 (13)
$n \geqslant 7.4\times10^6$	12 (19)
Total	64 (100)

3.4. Cytological analysis

In 59/64 (92%) cases, the diagnosis of small round cell tumours was achieved on cytomorphological data. The morphology was considered as typical of neuroblastoma with the presence of neuropil and neuroblastic cells or consistent with neuroblastoma in the more undifferentiated forms. The undifferentiated forms represented 30% of cases. The morphology needed to be correlated with immunocytochemistry data to achieve a definite diagnosis in the undifferentiated forms. In 5/64 (8%) cases, cytological analysis could not be performed for technical reasons: sample damage ($n=1$) and loss of smears ($n=2$) or absence of any tumour cells on smears ($n=2$). In these 5 cases, the diagnosis of neuroblastoma was subsequently achieved on percutaneous core biopsy of the primary in one case of localised ganglioneuroblastoma, on surgical biopsy in another localised neuroblastoma and on BM aspirates in the 3 remaining stage 4 neuroblastoma patients.

3.5. Immunocytochemistry

The median number of aspirated cells was 4×10^6 (range: 0–58×10^6). The mean percentage of tumour cells was 60% (range: 0–100%). The median number of tumour cells was 2.3×10^6 (range: 0–40.6×10^6). Table 1 details the amount of tumour cells collected with FNAC. Immunocytochemistry was evaluated from the material obtained by FNAC of the primary tumour in 56/64 cases (88%). In the 8 cases where FNAC did not provide enough tumour cells for immunocytochemistry, this analysis was performed from pleural fluid collections developed secondarily in aggressive stage 4 ($n=2$), bone marrow aspirates ($n=1$) or on smear preparations from surgical biopsy material ($n=2$) or percutaneous core biopsy ($n=1$). In one newborn child with dumbbell neuroblastoma, FNAC provided enough material for cytological study and *N-Myc* analysis, but not for immunocytochemistry and the histological diagnosis of neuroblastoma was eventually confirmed at operation following neo-adjuvant chemotherapy. In the last case, the histopathological diagnosis of neuroblastoma was assessed on percutaneous core biopsy and no immunocytochemistry was performed.

3.6. Molecular biology

N-Myc amplification was evaluated from the material obtained by FNAC of the primary tumour in 46/64 cases (72%). Two types of problems were encountered during molecular analysis in 16 cases: either a quantitative problem (a low number of cells were aspirated) or a qualitative problem (many cells were present but with a low percentage of tumour cells). In these cases, molecular analysis was incomplete or not possible, depending on the amount of tumour cells available in the laboratory. Technical problems occurred with the two additional samples: in one sample, tumour cells were numerous but altered during the transfer to the laboratory, while in the other the tubes broke during centrifugation. In these 18 cases, *N-Myc* amplification was assessed in 11 cases from surgical biopsy ($n=5$), BM aspirates ($n=3$), percutaneous core biopsy ($n=1$) or pleural fluid aspiration ($n=2$). In 7 cases, material for *N-Myc* assessment could not be obtained: 1 patient presented an acute onset of hypertension during surgical biopsy and the operation was suspended, 6 patients started treatment without additional procedures for evaluation of prognosis, in 3 cases because they presented a very poor status and 3 others were treated prior to 1990 when *N-Myc* was not required for treatment stratification. Table 2 correlates the extent of neuroblastoma and the feasibility of *N-Myc* and immunohistochemistry examination using cells extracted by FNAC. The best performance rate of FNAC for the sampling of tumour cells was observed in stage 4 neuroblastoma, where 70% were suitable for *N-Myc* analysis and immunocytochemistry examinations.

Table 2
Feasibility of laboratory analysis on FNAC according to the extent of neuroblastoma

Extent of disease	No. patients n (%)	Cytology n (%)	Immunochemistry n (%)	*N-Myc* n (%)	Immunohistochemistry and *N-Myc* n (%)
Localised	20 (31)	18 (90)	17 (85)	14 (70)	13 (65)
Metastatic >1 year	37 (58)	34 (92)	32 (86)	28 (76)	26 (70)
Metastatic <1 year	7 (11)	7 (100)	7 (100)	4 (57)	4 (57)
Total	64 (100)	59 (92)	56 (86)	46 (72)	43 (67)

FNAC, fine needle aspiration cytology.

Evaluation of the partial del 1p from FNAC samples was initiated in 1992. The number of tumour cells was sufficient for this analysis in 24/47 cases (51%) in addition to the other laboratory investigations (cytology, immunochemistry and *N-Myc* analysis).

4. Discussion

According to the INSS criteria [1,17] "an unequivocal pathologic diagnosis from tumour tissue (...) or bone marrow (...) unequivocal tumour cells and increased urine or serum catecholamines or metabolites" are required for the diagnosis of neuroblastoma. In addition, the INSS favours the attainment of "biologic characterisation of neuroblastoma cells (histopathology, immunophenotype, *N-Myc* gene amplification and DNA index) for diagnosis and prognostication". Until recently, guidelines in some cooperative groups recommended initial excisional surgery of the primary tumour, regardless of the stage, in the initial management of neuroblastoma. The gross resection or total removal of the tumour enabled histopathological diagnosis and prognostic evaluation with the Shimada and/or the Joshi classification [2,18]. Owing to concerns about morbidity and mortality in extremely ill children with advanced disease [19–23], other cooperative groups have recommended avoiding aggressive initial surgery in advanced unresectable neuroblastoma. As a consequence, initial tumour samples from the primary or metastatic sites have become crucial for definite diagnosis and analysis of prognostic markers, since chemotherapy may induce tumour changes and alter prognostic prediction in delayed biopsies [24]. During the last two decades, limited open biopsy has become an alternative to aggressive surgery in children with advanced or unresectable neuroblastoma. It is agreed that this approach is likely to reduce the risk of perioperative morbidity and to allow earlier initiation of chemotherapy. Data on the reliability of surgical specimens with regard to biological studies in neuroblastoma are missing. This prevents the accurate comparison of FNAC with surgical biopsies in the population of advanced localised neuroblastoma and metastatic neuroblastoma. A trend emerged during the 1990s towards obtaining material at presentation for histopathological diagnosis and biological analysis by percutaneous core biopsy [25]. Studies reported discordant results ranging from a 37–70% yield of *N-Myc* information from percutaneous core biopsy [25,26] and a 5% rate of major complications associated with this technique [25]. Furthermore, a risk of tumour seeding on the track of the needle has been reported following core biopsy [27]. Nevertheless, percutaneous FNAC of deeply located lesions has become widely used in the diagnosis of adult malignancy since the beginning of the 1980s. FNAC has become progressively accepted as a useful tool in the diagnostic work-up of various neoplastic masses in the paediatric age group [28–39]. However, the use of FNAC was limited in cases of suspected neuroblastoma because cytomorphology alone [29] was unable to differentiate neuroblastoma from other 'round blue cell' tumours (such as Ewing's sarcoma, Askin's tumour, nephroblastoma or rhabdomyosarcoma). Moreover, FNAC was unable to provide any histological information for the Shimada classification which was the unique prognostic parameter available. In the late 1980s, the development of immunocytochemistry allowed a more accurate diagnosis of neuroblastoma and the differential diagnosis of neuroblastoma from other round blue cell tumours [40]. Specific immunocytochemical stainings proved to be useful firstly for diagnostic information, such as the expression of neurone-specific enolase (NSE) [41], synaptophysin [42], ganglioside GD2 [15], neural cell adhesion molecule (NCAM) [43] and chromogranin A (CGA) [44] and secondly for prognostic evaluation such as expression of CD44 [9] and TRK-A [12]. The genetic study of tumours is providing important prognostic information, such as the integrity of chromosomes, especially chromosome 1 [7,8] and *N-Myc* oncogene amplification [3–5], with a high *N-Myc* copy number indicating a more aggressive form of neuroblastoma [6]. These laboratory techniques have initially been developed for application in BM samples in stage 4 neuroblastoma. The new possibilities from the laboratory prompted us to test their application in deeply sited primary neuroblastoma in which complete initial removal was clinically not indicated. The preliminary results of the series, presented in 1990 [13], prompted us to conduct the study on a larger number of cases. There have been several reports on the usefulness of FNAC in neuroblastoma. However, these series were either small [29,45] or heterogeneous in the technique used, e.g. *ex vivo* sampling [46]. Moreover, these series analysed together superficial and deeply located tumours [39], resectable and unresectable neuroblastoma, where difficulties for sampling and treatment stratification would differ. To the best of our knowledge, the presented series is the largest and most homogeneous reported on FNAC in deeply located unresectable neuroblastoma and the first which has analysed biological prognostic markers from *in vivo* FNAC sampling. Our series highlights several advantages for using FNAC in neuroblastoma.

FNAC is a safe tool with no complications observed in 64 patients with advanced untreated neuroblastoma. The presented series shows that FNAC could be performed safely in babies, in neuroblastoma encasing retroperitoneal vessels and for sampling small hepatic nodules in Pepper's syndrome. This represents an advantage over more invasive sampling techniques. A percentage of 5% acute complications has been

reported with percutaneous core biopsy [25] and complications of neuroblastoma surgery have been well described [19–23]. The importance of experience of any specialist involved in the multidisciplinary approach of paediatric oncology is clear. This is also true for the radiologist obtaining the samples and all those involved, i.e. the quality of the sample, the handling of the specimen to provide excellent preparation, the expertise of the pathologist performing the interpretation and above all good coordination between the specialists. Moreover, the procedure of sampling described in our series is well established in many malignancies and should be easy to set-up in any centre dealing with paediatric oncology.

FNAC saves time compared with surgical limited biopsy or classic gross total excision which may take up to one week to schedule. Moreover, surgical procedures are likely to delay the initiation of chemotherapy by a further week because of wound-healing and the risk of postoperative bowel obstruction. In most cases, FNAC was performed within the 48 h after admission. This enabled us to initiate chemotherapy within a few hours. From a financial point of view, it has been demonstrated that percutaneous biopsy of suspected abdominal malignancy provides substantial healthcare cost saving when compared with surgical biopsy [47]. This parameter was not included in our prospective study in 1987, but it should be noted before making recommendations on future protocol studies in neuroblastoma.

FNAC provided a reliable diagnosis in most cases. The conjunction of morphological analysis and minimal immunocytochemistry analysis (Anti-CD56+, Anti-GD2+ and Anti-CD45−) enabled us to achieve a diagnosis of neuroblastoma with FNAC in the majority of patients (88%). This diagnosis was confirmed in every case by subsequent histopathological analysis at the time of operation, following initial chemotherapy.

FNAC provided major prognostic information in most cases. Our results showed a 72% yield of information on *N-Myc* from percutaneous FNAC. Problems did arise when the number or percentage of tumour cells was low in the sample. Tumour differentiation may play a role in the amount of extracted cells: the more mature the tumour, the more difficult the aspiration. Correlation between the number of extracted cells and the histopathological subtype was impossible to assess because children underwent surgical removal after chemotherapy, which may modify tumour differentiation [20]. In an attempt to correlate the cytological grade of differentiation and the amount of aspirated cells, we found a trend for more differentiated tumours in the group of unsuccessful or partly successful sampling. Moreover, the good results for tumour sampling (Table 2) were achieved in metastatic neuroblastoma patients, who usually have a poor clinical status and should benefit from any alternative to aggressive surgical procedures. We focused our study on the ability of FNAC to provide *N-Myc* analysis. However, del 1p ploïdy may also become practical parameters of prognostic value, even though at present these factors do not influence therapeutic decisions in any international protocols. In our series, *N-Myc* amplification was analysed by Southern blotting that required more than 1.5×10^6 cells containing more than 50% malignant cells. However, new micromethods such as FISH analysis [16,48,49] now allow the analysis of *N-Myc* as well as del 1p using only 1×10^5 cells. It is likely that FISH should increase the performance of FNAC in neuroblastoma for *N-Myc* analysis, as well as for any new biological parameters.

Tumour heterogeneity is a difficult challenge regarding both the histopathological and the biological analysis in neuroblastoma. In theory, only the total surgical removal of the tumour is able to provide an exhaustive histopathological analysis, especially in the nodular form, and surgically limited biopsy or percutaneous core biopsy may be misleading in such cases. Moreover, most oncological teams rely on the *N-Myc* amplification analysis to achieve treatment stratification. The presented series showed the reliability of FNAC to provide sufficient material for the *N-Myc* amplification analysis in advanced neuroblastoma. With the recent evidence that neuroblastoma can present with biological heterogeneity [50], a limited tumour sample could be misleading whatever the method of sampling used, i.e. limited surgical biopsy, percutaneous techniques or BM sampling. However, it has not yet been demonstrated that such heterogeneity is of prognostic significance.

In summary, we suggest FNAC should be the first strategy in neuroblastoma which is not totally resectable because it provides the required information for treatment stratification, rapidly and safely, in 72% of cases. This success rate is at least as good as the reported results with core or surgical biopsies. Percutaneous core biopsy presents a higher risk of complications and should, therefore, be limited to immediate complementary sampling in cases of inadequate counts of tumour cells from FNAC. Due to their morbidity and mortality risks, their costs and the delay before the onset of chemotherapy treatment, we suggest that surgical procedures for neuroblastoma that is not totally resectable should be limited to the cases of failure of percutaneous sampling.

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