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Acknowledgements—Supported by C.N.R., Acro, grant No. 94.01196.PF39 and by Associazione Italiana per la ricerca sul cancro (AIRC).

Pergamon

European Journal of Cancer Vol. 31A, No. 4, pp. 499–504, 1995

0959–8049/95 $9.50+0.00

0959-8049(95)00043-7

Gene Expression and Protein Localisation of Calcyclin, a Calcium-Binding Protein of the S-100 Family in Fresh Neuroblastomas

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Calcyclin gene, a Ca^{2+}-binding protein with homology to S-100, has been found to be expressed at different levels in leukaemic cells and in other tumour cells. We recently reported the expression of the gene in human neuroblastoma (NB) cell lines, and suggested a possible role of calcyclin in cell differentiation. To extend our findings, we investigated the expression of the gene in NB cells induced to differentiate by retinoic acid (RA), using the reverse transcriptase-polymerase chain reaction (RT-PCR) technique. Time-course experiments employing LA-N-5 cells showed that calcyclin mRNA appeared 2 h after RA treatment, long before the cells were blocked in the G1 cell-cycle phase and before the neurite-like structures outgrew from the cell bodies. This suggests the involvement of the gene in the early phase of cell differentiation. Furthermore, we investigated mRNA expression in a series of fresh neuroblastomas. NB tumours showed a heterogeneous pattern of calcyclin expression, although calcyclin seemed to be expressed more frequently in cases with a favourable Shimada histology. We also studied the expression of the protein in formalin fixed and paraffin embedded tissues, by using a specific anticalcyclin antibody. The protein was detected in stromal cells which characterise a more mature histological type, and in nerve sheaths, whereas neuroblasts were negative. The tissue that expressed calcyclin protein showed a Schwann-like differentiation and, unlike S-100 protein, calcyclin was expressed in the perineurium.

Key words: neuroblastoma, calcyclin, S-100, gene expression, retinoic acid, cell differentiation, polymerase chain reaction

Eur J Cancer, Vol. 31A, No. 4, pp. 499–504, 1995

INTRODUCTION

CALCYCLIN BELONGS to the large S-100 protein family. The product of the calcyclin gene [1, 2] is a calcium (Ca^{2+})-binding molecule [3, 4] that shows partial homology to the α and β subunits of S-100 protein [5, 6]. Calcyclin mRNA expression increases during the transition G0 to S cell-cycle phases of serum-stimulated quiescent fibroblasts [2]. Calcyclin expression has been found in both normal human and animal tissues. The highest levels have been observed in fibroblasts, in epithelial cells [7] and in decidual cells [8]. The expression of the gene has been shown to be deregulated in certain human leukaemias, in colon cancer [9, 10], and in some melanoma cell lines [11], whereas its increased expression has been associated with the metastatic activity of *ras*-transformed fibroblasts [12]. Together these findings suggest that calcyclin plays a multiple role in normal and tumour cells. We found calcyclin mRNA expression in human neuroblastoma (NB) cell lines [13], and showed that the gene is mainly expressed in I-type rather than in N-type cells. Neuroblastoma cell lines show at least three cell-type morphologies: N, neuroblast; I, intermediate; and S, substrate-adherent. Each type has distinct biochemical characteristics [14], and the different expressions of calcyclin in N- and I-type cells provide additional evidence for their different molecular composition.

Despite their different morphologies, almost all NB cell lines are sensitive to retinoic acid (RA) an agent that induces morphological cell maturation. The morphology of NB cells treated with RA changes and long neurites outgrow from the cell bodies [15]. We studied the induction of calcyclin mRNA during cell differentiation, by using the reverse transcriptase-polymerase chain reaction (RT-PCR) technique.

S-100 protein, the parental protein of calcyclin, is also induced during neuroblastoma cell differentiation [16]. This protein is a marker for glial and Schwann cells [17], and S-100 positive cells are associated with a good prognosis in neuroblastoma patients [18]. Because of a possible similar function of calcyclin, we used both the northern blot and RT-PCR techniques to study gene expression in a series of fresh NBs belonging to the Tissue Bank of the Italian Paediatric Cancer Research Group [19]. Finally, to identify the distribution of calcyclin protein in the tumour, we used an anticalcyclin antibody to detect the protein expression in both stroma-rich and stroma-poor neuroblastoma.

MATERIALS AND METHODS

Cell lines and tumour tissue

Human NB cell lines LA-N-5 and SK-N-BE(2)C, gifts from M. Seeger (Los Angeles, U.S.A.) and V. Ciccarone (New York, U.S.A.), respectively, were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2% glutamine, 1% non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin. For cell differentiation, the medium was replaced every 2 days in both untreated and RA-treated cells. We used RA at a 10 μM final concentration.

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Tissue from 23 tumours was immediately frozen in liquid nitrogen after surgery. NBs were classified according to Shimada and associates [20]. The Shimada criteria classify tumours into favourable and unfavourable histologies. A stroma-rich neuroblastoma (SRNB), in which immature cells are uniformly distributed, belongs to the favourable histology and can exhibit growth of a Schwann-like spindle cell stroma. A stroma-poor neuroblastoma (SPNB), in which the Schwann-like stroma is virtually absent, is classified as unfavourable histology. The karyorrhexis index and the age of patients are also taken into account in Shimada's classification. Clinical staging followed the one proposed by Evans and associates [21].

RNA isolation and analysis

Each frozen tumour biopsy (23 samples from untreated patients) was mechanically reduced to powder in a mortar containing liquid nitrogen. Total RNA was extracted from the tumour and the cell lines as previously described, according to the guanidine isothiocyanate phenol/chloroform/isoamyl alcohol method [22, 23]. Briefly, a guanidine suspension was extracted with phenol/chloroform/isoamyl alcohol (50:50:1). After precipitation in cold ethanol, RNA was washed twice with 70% ethanol, dried and resuspended in water at an optimal concentration. RNA (10 μg/lane) was electrophoresed on a 1.2% denaturating agarose gel containing 2.2 M formaldehyde in 20 mM morpholinopropansulphonic acid, 5 mM sodium acetate, and 0.5 mM EDTA buffer at pH 7.0. Then RNA was blotted on to Hybond C extra membrane (Amersham, U.K.) and the filter was baked at 80°C for 2 h [24]. The calcyclin probe (clone hp2A9) containing the second and third exons of the gene, was provided by S. Ferrari of the University of Modena. The 0.7 kb insert of *Bam*HI was separated by electroelution [24]. Prehybridisation and hybridisation were performed as previously described [23]. Autoradiography was performed at −80°C overnight with one intensifying screen. After stripping the calcyclin probe, the amount of RNA loaded into the gel filter was checked by hybridising the filter with the housekeeping gene, *GAPD*. Probes were labelled with [α-^{32}P] dCTP (3,000 Ci/mmol, Amersham, U.K.), by the random primer method [25] (Multiprime labelling kit, Amersham, U.K.).

Reverse transcription and PCR

The preparation of the first strand of cDNA from total RNA for subsequent use in PCR was obtained by employing the first-strand cDNA synthesis kit (Clontech, California, U.S.A.). In short, 1 μg of total RNA was incubated with 20 μM of oligo(dT)$_{18}$ primer at 70°C for 2 min in a final volume of 13.5 μl of diethyl pyrocarbonate (1:1000) (DEPC) water. Then the mixture was incubated for 1 h at 42°C in a total reaction volume of 20 μl containing 5 X reaction buffer, dNTPs Mix (10 mM each), recombinant RNAse inhibitor and M-MLV reverse transcriptase. The cDNA synthesis reaction was stopped by heating at 94°C for 5 min, and diluted to the final volume of 100 μl by adding 80 μl of DEPC-treated water. The sequences of the pair, which we chose for the subsequent amplification by PCR, were:

sense 5′–AAGCACACCCTGAGCAAGAAGG–3′;
antisense 3′–GCCTTGGCTTTGATCTACAATG–5′.

The primers were synthesised with the DNA–SM Beckman Instrument. The sense primer recognises exon 2 (728–750) and the antisense recognises exon 3 (1259–1281); the product of RT-PCR is a fragment 181 bp long. After first-strand cDNA

synthesis, the primer pairs were added to the reaction mixture, and PCR amplification was performed in a thermal cycler (Perkin-Elmer-Cetus, Norwalk, Connecticut, U.S.A.), according to the following protocol: initial denaturation at 94°C for 2 min was followed by 35 cycles of 30s denaturation, annealing the primers at 62°C for 30 s and extending the primers for 1 min at 72°C. A further 10 min extension at 72°C was performed to extend any remaining single-strand product. The PCR products were examined by agarose gel electrophoresis, stained with ethidium bromide and photographed in UV light.

Detection of calcyclin and S-100 protein by immunohistochemistry

The immunohistochemical study was performed on paraffin embedded, formalin fixed tissues representative of the different clinical stages, using the avidin–biotin–peroxidase complex (ABC) technique [26] for detection of either S-100 antibody (Dakopatts, Copenhagen, Denmark) or calcyclin antibody [4].

RESULTS

Early detection of calcyclin mRNA during neuroblastoma cell differentiation

To detect the lowest level of calcyclin expression during RA-induced cell differentiation, we used the very sensitive RT-PCR method. Time-course experiments using LA-N-5 cells, which do not express calcyclin, showed that mRNA was detectable 2 h after RA treatment, when the band of the RT-PCR product is clearly visible in the agarose gel (Figure 1). During this time, the cell morphology, observed using a phase-contrast microscope, did not change, and only after 48 h of RA treatment did neurite-like protrusions outgrow from the cell bodies, with full morphological differentiation achieved after 96 h.

RNA calcyclin expression in fresh tumours

On northern blot, calcyclin mRNA was found variably expressed in 15 of the 23 NB samples (Table 1 and Figure 2) and barely detected (−/+ in Table 1) in 3 samples. The remaining 5 tumours were negative for calcyclin expression. To detect the presence of the lowest level of calcyclin mRNA, RT-PCR analysis was performed on northern blot negative samples whose RNAs were available. RT-PCR analysis was able to detect calcyclin mRNA in 2 of the 5 negative cases (samples 9 and 18). Sample 4, which showed a weak signal by northern blot, was also positive in RT-PCR. Finally, sample 15, to which a −/+ evaluation was assigned, was negative.

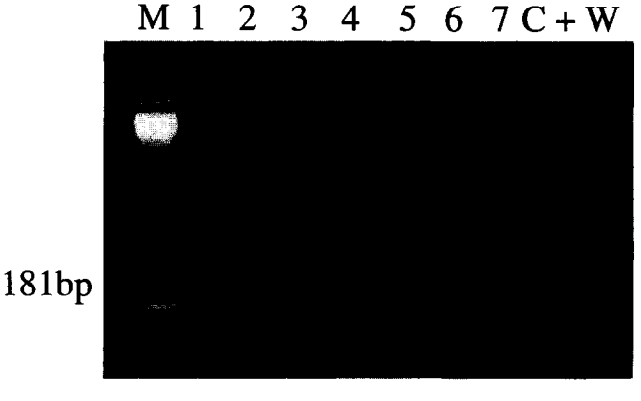

Figure 1. RT-PCR of the calcyclin gene mRNAs of RA-treated LA-N-5 neuroblastoma cells. The first lane shows the 123 bp DNA ladder molecular weight (M). From left to right, a time-course experiment is shown: 1, untreated cells; 2–7, 2, 4, 8, 12, 24, 48 h RA treatment respectively; C+, SK-N-BE(2)C, the positive control for calcyclin; W, primers without mRNA.

Results show that almost all neuroblastoma tissues express the gene at low or high levels. The majority of NBs were classified as stroma-poor (19/23) and undifferentiated (12/23) (Table 1). Calcyclin was expressed in 86% of NBs with a favourable Shimada histology and in 67% with unfavourable histology. Sample 22, characterised by a very low level of calcyclin mRNA, was considered negative. No correlation with clinical stages was found. We did not find any association of calcyclin expression with a higher patient survival: among children whose tumours expressed calcyclin, 50% (9/18) are still alive and disease-free, whereas 80% (4/5) of these patients whose tumour cells did not express the gene, were alive at the evaluation time.

Calcyclin and S-100 protein expression on tissue sections

Table 2 gives the results of the immunohistochemical study of calcyclin and S-100 proteins in seven representative cases, including 2 SRNBs and 5 SPNBs. Calcyclin positivity was observed in the neuromatous stroma (samples 8 and 17) of the two stroma-rich NBs. In two SPNBs, calcyclin protein was located in a few spindle cells in thin perivascular septa. Neuroblasts were negative in SRNB sample 17, and there was weak staining of only occasional ganglion-like neoplastic cells. The intensity of the stain was not correlated with mRNA expression. S-100 protein showed a very similar staining pattern, although it was much stronger than that of calcyclin protein. A striking presence of S-100 protein was seen within the large ganglion-like cells of SRNBs and in a few neuroblasts in two SPNBs samples. In addition, it was interesting to note that the calcyclin antibody stained only the perineurium (Figure 3a), whereas the S-100 protein antibody also stained the nerve bundles (Figure 3b).

DISCUSSION

Calcyclin, a Ca^{2+}-binding protein, has been found to be highly expressed in different tissues of nude mice and in certain human tumours [9–11]. In a previous study, we demonstrated that calcyclin mRNA was differently expressed in proliferating human NB cell lines, and that it was induced during neuroblastoma cell differentiation [13]. In the present paper, we extended our observations on calcyclin mRNA induction to RA-treated NB cells, and reported the results of the calcyclin gene expression in a series of fresh NB tissues.

Neuroblastoma cells are very sensitive to RA activity. This characteristic behaviour suggests that a failure of the cell differentiation pathway may be a general defect of NBs. After RA treatment, they show a dramatic morphological change, and assume a terminal differentiated phenotype: cells display stretching bodies, and long neurite-like structures protrude from the cell bodies, forming a complex neuronal network. Using the RT-PCR technique, we observed an early induction of the calcyclin gene. Specific transcripts were detected 2 h after treatment, long before the cells were blocked in the G1 cell-cycle phase and before their full morphological differentiation occurred. This early appearance of calcyclin mRNA suggests that the gene is involved in neuroblastoma cell differentiation [27]. This is of particular interest, as the gene might be associated with other genes, like *MYCN*, *FOS*, *C-HA-RAS*, and *C-ETS-1*, which have been found to play a role in neuroblastoma cell

Table 1. List of molecular and histological data of neuroblastoma samples (clinical stages are also given)

Sample	Stage*	Source†	Calcycin‡	Stroma	Morp§	MKI‖	Prog ¶	Outcome**
1	1	P	+++	Poor	Und	Under 100	F	A
2	1	P	+	Poor	Und	Under 100	U	A
3	1	M	−	Poor	D	Under 100	F	A
4	1	P	−/+(+)	Poor	D	Under 100	F	A
5	1	P	+	Rich	I	Under 100	F	A
6	2	P	−	Poor	Und	Under 200	F	A
7	2	P	+	Poor	Und	Under 100	F	D
8	2	P	++	Rich	I	—	F	A
9	2	P	−(+)	Poor	D	Under 100	F	A
10	2	P	++	Poor	D	Under 200	F	A
11	3	P	+	Rich	I	—	F	AWD
12	3	P	+	Poor	I	Over 200	U	D
13	3	P	+	Poor	Und	Under 100	U	A
14	4	P	+	Poor	D	Under 100	F	D
15	4	P	−/+(−)	Poor	Und	Over 200	U	A
16	4	P	+	Poor	D	Under 100	F	D
17	4	P	+++	Rich	I	—	F	D
18	4	M	−(+)	Poor	Und	—	U	D
19	4	P	−	Poor	Und	Under 200	U	D
20	4	P	++	Poor	Und	Under 100	F	A
21	4	M	+	Poor	Und	—	U	D
22	4S	M	−/+	Poor	Und	—	U	A
23	4S	P	+++	Poor	Und	Over 200	U	AWD

* According to Evans [21]; † P, primitive tumour; M, metastasis; ‡ Expression of calcyclin mRNA: none (−), barely detectable (−/+), low (+), high (++), very high (+++). Signs within parentheses indicate the results of RT-PCR of northern blot negative samples whose RNAs were available; § Morphology: Und, undifferentiated; D, differentiated; I, intermixed; ‖ Mitotic karyorrhexis index; ¶ Prognostic evaluation according to Shimada. F, favourable, U, unfavourable; ** Outcome: A, alive; AWD, alive with disease; D, dead.

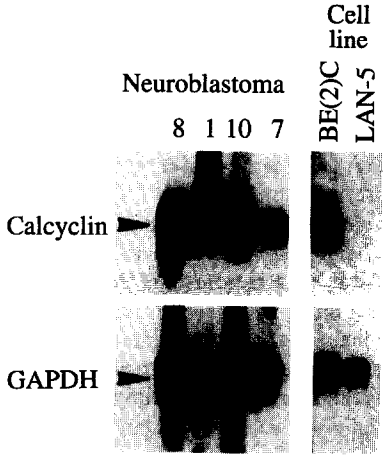

Figure 2. Expression of the calcyclin gene in neuroblastomas (samples 1, 7, 8 and 10). A representative northern blot analysis of calcyclin mRNA expression is shown. The size of calcyclin mRNA is 0.7 kb. After stripping the calcyclin probe, the filter was hybridised again with a control housekeeping gene, *GAPD*, to check the amounts of RNA loaded in all lanes. SK-N-BE(2)C, LA-N-5 human NB cell lines were used as positive and negative controls of calcyclin expression, respectively.

differentiation [28]. Studies by Thiele and colleagues [29] and our previous experiments using LA-N-5 cells [30] showed that *MYCN* expression decreased by approximately 50% 48 h after RA treatment. Our data indicate that calcyclin is rapidly activated before the decrease in *MYCN* oncogene expression. We recently studied the binding of proteins to the promoter region of the calcyclin gene during LA-N-5 cell differentiation [31], and found an increased binding of an AP-1-like element that was parallel to the increase in its transcription. RA may enhance calcyclin expression by activating the gene in trans so as to make the role of the gene remarkable for the differentiation of RA-treated LA-N-5 cells.

It is worth noting that S-100 protein has also been involved in NB cell growth and differentiation, and that S-100 acts as a neurotrophic factor [23]. We reported that RA is able to induce protein kinase C (PRKC) mRNA expression in NB cells 32. A link of calcyclin with PKC and its metabolism has also been described [33]. Therefore, it becomes increasingly evident that calcyclin, like other members of the S-100 family, is involved in the recruitment of Ca^{2+} and in signal transduction during NB morphological differentiation induced by RA.

To better understand the role of the calcyclin gene in neuroblastoma development, we studied its expression in fresh tissues. Our results showed that calcyclin mRNA was found variably expressed in NB specimens by northern blot analysis. Using RT-PCR analysis, we were able to detect calcyclin mRNA in 2 of the 5 negative cases studied by northern blot, thus providing evidence that, in some tumours, there may only be a few cells expressing calcyclin.

Table 2. Comparison between calcyclin mRNA expression and protein expression in 7 representative samples of neuroblastoma tissue

Sample	Stage*	Calcyclin mRNA†	Calcyclin Protein ‡	S–100 protein‡
1 SPNB	1	+++	n.d.	(+) Periv. stromal cells (+) Few neuroblasts
8 SRNB	2	++	(+) Neuromatous stroma (−) Neuroblast ganglion-like cells	(++) Neuromatous stroma (+) Ganglion-like cells
9 SPNB	2	—	(−)	(+) Periv. stromal cells (+) Few neuroblasts
17 SRNB	4	+++	(+) Neuromatous stroma (+) Nerve (−/+) Ganglion-like cells	(++) Neuromatous stroma (++) Perineurium (+) Ganglion-like cells
20 SPNB	4	++	(−)	(+) Periv. stromal cells
22 SPNB	4S	+/−	(+) Periv. stromal cells	(+) Periv. stromal cells
23 SPNB	4S	+++	(+) Periv. stromal cells	(+) Periv. stromal cells

* See Table 1. † See Table 1. ‡ Expression. n.d., not detected; −, none; −/+, weak positivity; +, positivity; ++, strong positivity. SPNB, stroma-poor neuroblastoma; SRNB, stroma-rich neuroblastoma; Periv., perivascular. The comparison between calcyclin protein expression and S-100 protein is shown for the same samples. Protein expression was detected by the immunohistochemical method described in the Materials and Methods section. RNA expression was taken from Table 1 and detected by northern blot analysis.

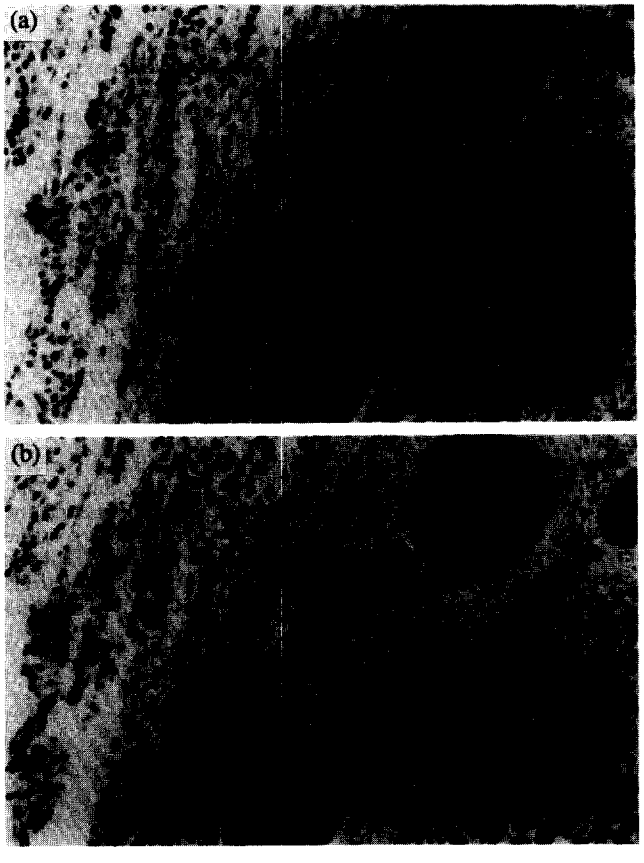

Figure 3. Two consecutive sections of the same inclusion stained with (a) calcyclin and (b) S-100 antibodies. (a) SRNB calcyclin positivity in neuromatous stromal cells and in perineurium. (b) Stronger SRNB S-100 positivity in spindle cells. Nerve bundles are stained whereas the perineurium is not stained.

Neuroblastoma shows a complex histological pattern, consisting of neuroblasts dispersed in an abundant stroma, or closely packed neuroblasts, the stroma being virtually absent. Immunohistochemical analysis demonstrated that calcyclin protein is expressed in stromal cells in both SRNBs and SPNBs, which show a Schwann-like differentiation. Particularly in SRNBs, the neuromatous stroma was positive, whereas undifferentiated neuroblasts were negative. SPNBs showed positivity in the thin fibrovascular septa. Our data support the hypothesis that the calcyclin gene is expressed in more mature neuroblastomas, and that it might be involved in cell differentiation. This is consistent with our observation that calcyclin is prevalently expressed in stromal cells and in cells with characteristics of a Schwann-like differentiation.

The presence of calcyclin transcripts, observed by northern blot and/or RT-PCR, showed no significant association with localised or metastatic stages. Approximately 69% (9/13) of patients who are alive and disease-free expressed calcyclin, as compared with 31% (4/13) of living patients whose tumours did not express the gene. However, these data are not sufficient to define calcyclin as a favourable marker in neuroblastoma patients.

Since calcyclin shows homology to the β subunit of S-100 protein, we compared the expressions of the two proteins in fresh tissues. The immunohistochemical study showed that the two proteins had different staining intensities but similar distributions in the stromal components of both SRNBs and SPNBs. However, calcyclin was expressed in nerve sheaths, but not in neuroblasts, and, unlike S-100 protein, it was detected in the perineurium.

In conclusion, our results indicate that calcyclin may play a role in neuroblastoma cell differentiation and that this protein, like S-100 protein, could be used to better characterise the cells forming the tumour.

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Acknowledgements—The authors wish to thank Dr B. De Bernardi and Dr A. Garaventa for their co-operation in providing clinical data. They are also grateful to Dr Mariapaola Dentone for editing the manuscript. This work was developed within the framework of the research programme on neuroblastoma promoted by the Italian Pediatric Cancer Research Group (IPCRG) and supported by Associazione Italiana Ricerca sul Cancro (AIRC), Project No. 11 CNR-ACRO (Consiglio Nazionale della Ricerca) and UTIS.