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International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

Collaborative Research Programme

TERMINAL EVALUATION REPORT

Part 1

Title of Project

PAPILLOMAVIRUS, CELLULAR ONCOGENES AND HUMAN CANCER

Keywords:

UNIDO contract # 92/038 **ICGEB ref. #:** CRP/ MEX91-01

Project initiation: MARCH 17, 1992 **Project termination:** MARCH 17, 1995

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Abstract:

The long-term purpose of this proposal in to gain understanding of the molecular mechanisms involved in uterine cervix carcinoma among mexican women. The polymerase chain reaction (PCR) was employed to determine the fraction of tumors presenting high-risk HPV sequences and the percentage of clinically normal women containing these sequences. Previous results from our group obtained using Southern blot indicated that oncogenic human papillomaviruses (HPVs) are associated to a relatively low percentage of mexican uterine cervix tumors. Now, using PCR in 125 squamous cell carcinomas (clinical stage II or III) we have detected high-risk HPV sequences in 78.4% of the patients, most of them (49%) related to HPV-16. In addition, 100% (8/8) of cell lines derived from uterine-cervix carcinomas contained high-risk HPV sequences (most of them belonging to HPV-18).

Cellular oncogenes could cooperate with HPV in the development of cervical carcinomas: we have detected frequent c-myc alterations in invasive carcinomas. We recently expanded this observation for both premalignant lesions and cell lines derived from cervical carcinomas. In addition we have detected ras mutations and Rb gene alterations in cervical lesions. By both mRNA in situ hybridization and immunohistochemistry we determined that uterine-cervix carcinomas are extremely heterogeneous in relation to myc and pRB expression and surprisingly we observed a frequent inverse relationship between the expression level of these genes.

We have determined that anti-oncogenic proteins (p53 and pRB) are capable of suppressing viral early gene expression. In addition we have determined the mechanism by which viral encoded E2 protein represses early HPV transcription. In the contect of the natural viral promoter the E2 protein inhibited viral transcription in vivo. However this repression was released and transformed in activation of E6/E7 transcription when we increased the distance between the TATA box and the proximal E2 binding site.

With regard to the humoral immunoresponse to HPV early proteins in malignant and premalignant cervical lesions, we detected antibodies against HPV E7 oncoprotein in malignant lesions containing elevated copy number of viral sequences. Surprisingly, we detected antibodies against ras oncoprotein. Antibodies against HPV-16 oncoprotein E7 were detected in a higher proportion of cervical cancer patients compared to patients with cervical intraepithelial neoplasia or normal individuals. A higher prevalence of sera with antibodies to p21 ras was observed in patients with premalignant lesions compared to healthy individuals.

Part 2

OBJECTIVES/METHODOLOGY
(proposed at the time of the submission of the research proposal)

The main goal of this project was to understand the participation of high-risk HPVs and cellular genes in uterine-cervix cancer development.The specific objectives were:

A) Molecular epidemiology studies on the prevalence of high-risk HPVs using the PCR methodology in different samples:

1.- Invasive uterine-cervix carcinomas

2.- Scrapes from clinically normal women.

3.- Cell lines derived from cervical carcinomas.

B) Studies on cellular oncogenes and tumor suppressor genes:

1.- Frequency of c-myc alterations in precancerous and invasive carcinomas as well as in cell lines derived from cervical tumours.

2.- Same for ras, Rb and p53.

3.- Expression level and intracellular localization of the corresponding proteins and mRNAs using immunohistochemistry and in situ hybridization methodologies.

C) Viral early gene expression regulation by:

1.- p53

2.- pRb

3.- Viral E2 protein

D) Studies on the humoral immunoresponse to viral and cellular oncogenic products.

RESULTS
(compare against the set objectives)

A. Molecular epidemiology studies on the prevalence of high-risk HPVs using the PCR methodology in different samples:

1.- Invasive uterine-cervix carcinomas.

In 125 squamous cell carcinomas (the majority of them belonging to clinical stage II or III) we demonstrated that about 80% of the samples contain high-risk HPVs, with48.8%, 18.4% and 11.2% for HPV-16, HPV-18 and HPV-30s, respectively. We (and others) have strong evidence that about 10-20% of mexican high-risk HPVs could be represented by viral subtypes.

2.- Scrapes from clinically normal women.

So far, we have studied 120 clinically normal women using PCR (β globin positive samples) and found that approximately 20% of them presented high-risk HPV DNA sequences, as compared to about 40% in women presenting different types of premalignant cervical lesions.

3.- Cell lines derived from cervical carcinomas.

In all cell lines studied so far (8/8) we were able to detect high-risk HPV DNA. Surprisingly, most of them contained HPV-18 in an integrated state.

B.- Studies on cellular oncogenes and tumor suppressor genes:

1.- Frequency of myc alterations in precancerous and invasive carcinomas as well as in cell lines derived from cervical tumours.

We have determined that c-myc is frequently altered in uterine-cervix carcinoma. About 80% of cervical malignant tumours present c-myc amplification and/or rearrangement. In addition, we determined that c-myc alterations are less frequent in precancerous lesions (22%) than in cervical carcinomas. Cell lines derived from uterine-cervix tumours presented frequent (5/8) c-myc alterations.

2.- Frequency of ras, Rb and p53 alterations in the above samples. Preliminary results indicated that a low percentage of cervical intraepithelial neoplasias (CIN), invasive cervical carcinomas or cell lines derived from these present gene alterations. For

example, in one study, only 5% of CIN patients presented sera antibodies against ras protein. We recently showed that ras mutation was a common event in precancerous lesions (CIN) obtained from these patients. With regard to Rb tumor suppressor gene, two of eight cell lines analyzed by Southern blot showed gene alterations.We are currently sequencing oncogenes and tumor suppressor genes in CIN and cervical carcinomas.

3.-Expression level and intracellular localization of oncogenic and anti-oncogenic proteins. Employing Northern blot, Western blot, in situ hybridization for mRNA and immunohistochemistry, in both premalignant or malignant lesions, we observed c-myc overexpression frequently correlated with c-myc gene alterations. Interestingly, an inverse relation was obtained when c-myc and pRb expression was compared.

C.- Viral early gene expression regulation by:

1.- p53. We recently demonstrated using transient transfection experiments that p53 alters transcription of the long control region (LCR) of HPV18. To determine whether wild-type p53 influenced transcription of the HPV-18 LCR, normal human keratinocytes or C-33A cells (which contain an endogenous p53 with 273Arg to Cys mutation) were cotransfected with the wild-type p53 expression plasmid pZIP-Neo-p53S and the CAT-reporter plasmid p18/42 containing the HPV-18 LCR. Overexpression of wild-type p53 reduced the activity of the HPV-18 LCR in both the wild-type p53-containing keratinocytes and the mutant p53 containing C-33A cells. Transcriptional repression was observed in normal human keratinocytes by both wild-type and mutant p53.

2.- pRb. It is known that the protein product of the retinoblastoma tumor suppressor gene (pRb) can form complexes and inactivates the transcriptional factor E2F or the c-myc protein in vitro. To determine whether pRb regulates both cellular (c-myc) and viral (HPV LCR) promoter activity in vivo, Myc-CAT or HPV LCR-CAT chimeric expression plasmids were generated and cotransfected with a pCMV-RB expression plasmid. pRb repressed both myc and LCR transcription but not SV40-CAT. Transcriptional repression induced by pCMV-RB was relieved by addition of pSV2-E7 and was dependent on an LCR E2F site. Moreover, immunohistochemical assays indicated that in cervical intraepithelial neoplasia lesions, an increased pRb expression correlates with decreased c-myc oncogene expression. These results suggest that pRb can negatively regulate c-myc transcription in vivo in both normal tissue or early cervical lesions. In HPV induced invasive cervical carcinomas where viral DNA is integrated expressing its oncoproteins, pRb could be complexed by HPV E7

oncoprotein releasing the repression effect and promoting cell growth.

3.- Viral E2 protein.

We analized the natural position of E2 binding sites (E2BS) in HPVs from cutaneous and mucosal lesions. Interestingly, we found that the positions differ between these two viral groups. HPVs usually found in lesions of the genital mucosa have two E2BSs very close to the E6/E7 start site, whereas those frequently found in lesions of the skin have their most proximal E2BS more than 100 bp from the E6/E7 start site.

To determine if genital and cutaneous HPV early promoters are differentially regulated by the E2 protein, we cotransfected HeLa cells with a construct containing the HPV-20 LCR linked to the CAT gene and pC59 (expression plasmid that codes for the BPV-1 E2 protein) or carrier DNA. As a control we cotransfected p18/42 (posses the natural HPV-18 P105 promoter) and pC59; we observed that, as expected, the cutaneous HPV-20 construct is activated whereas the HPV-18 construct is repressed by the E2 protein.

In order to separate the proximal E2BS from the TATA box on the natural context of the HPV-18 LCR we constructed two plasmids (pCG1 and pCGK) that increased the distance between these sites. When C-33A or SW13 cells were cotransfected with reporter plasmid pCG1 (9bp between the proximal E2BS and the TATA box) and expression plasmid pC59, the promoter activity of pCG1 was repressed by 58%. A notable difference does exist when this value is compared to 90% repression obtained when BPV-1 E2 acts on the p18/42. This observation suggests that even at a distance of 9bp between the TATA box and E2BS, the E2 protein is capable of interfering with transcription initiation complex formation. The presence of the trans-activation domain does not prevent full length E2 protein from repressing in vivo transcription of the HPV-18 P105 promoter containing an additional 6 nt between the proximal E2BS and the TATA box.

On the other hand, when we evaluated the effect of BPV-1 E2 protein on the promoter activity of the pCGK construction (12 bp distance between TATA and E2BS) we observed an increase in the activity of approximately 50% in the presence of BPV-1 E2. This result suggests that when the distance between E2BS and TATA sequence is increased to 12 bp, E2 protein and TBP can bind in vivo simultaneously to the promoter; the E2-TBP-TATA box complex could be a stronger transcriptional activator

than the TBP alone, perhaps by increasing the binding affinity of TFIID for the promoter. The above experiments indicated that the same protein can function in vivo as both an activator or a repressor of transcription. This provides a further understanding of the mechanism by which the E2 transregulator can function in the natural context of the HPV-18 early promoter and by which the transcriptional regulation of genital and cutaneous HPV's differs.

D.- Studies on the humoral immunoresponse to viral and cellular oncogenic products.

For the detection of antibodies against p21 ras and HPV-16 early proteins in sera from patients with uterine cervical lesions, we tested 43 sera from patients that presented different levels of cervical lesions (most of them premalignant) and also analyzed 34 sera from normal individuals that were used as controls. The sera were tested for the presence of antibodies against HPV-16 E4 and E7 proteins and p21ras. This was done by Western blot analysis, using the cII and MS2 recombinant proteins produced and isolated from bacteria. The results for the 43 sera obtained from patients indicated that they presented different reactivity patterns against the fusion proteins; about 44% (19/43) of them were positive for E4 (13.6KD), and 20% (9/43) were positive for ras. In 9% (4/43) of the patients we found antibodies against E7 corresponding to 28.5% (2/7) of the invasive uterine cervix carcinoma patients studied and about 8% (1/12) of the CIN III patients. The possibility that the antibodies were recognizing the lambda bacteriophage coded cII region is unlikely since MS2 fusion proteins corroborated our results and only 2% (1/43) of the sera reacted to both cIIE7 and cII-Ha-ras in the same paper strip; this serum was later shown to contain antibodies against p21ras. From all these 43 sera, we found that 24 of them have reacted at least against one of the fusion proteins used as antigens. Only one serum (1/34) from normal individuals presented antibodies against ras, suggesting that our system was specific.

To determine ras antibody concentration and to confirm that the sera positive for ras protein was real and not an artefact, we did dilutions from two different sera that gave strong response against cII-Ha-ras and used a serum that was negative for ras protein, but positive for HPV-16 E7 oncoprotein, as internal control. The response of sera from patient 33 (positive for ras and E7) and 36 (positive for ras only) against the ras protein was evident even after a 1/720 dilution. We also used cellular c-Ha-ras protein as antigen to test these sera for the presence of antibodies against p21ras protein; both patient 33 and patient 36 sera showed a strong response against cellular p21ras,

whereas sera from normal individuals (N10, N11) did not contain these antibodies, suggesting that the immune reactions were directed against ras and not the cII portion of the molecule. Corresponding dilutions of rabbit polyclonal antibodies against ras proteins and purified human IgG were also employed as positive and negative controls, respectively, to demonstrate that the Western blot system was specific.

To further investigate the immuno-response against p21ras found in sera from patients with different cervical lesions, we did competition assays with purified recombinant ras protein (cII-vHa-ras or cII-cHa-ras). We used as antigen either cII-vHa-ras, cII-cHa-ras or c-Ha-ras obtained from cells overexpressing p21ras. In particular, we wanted to compare the response for normal c-Ha-ras and for activated Ha-ras proteins.We performed a Western blot assay, using the fusion cII-cHa-ras or cII-vHa-ras proteins as nitrocellulose filter immobilized antigens; the competition of ras antibodies present in serum 36 (positive for ras) was done by mixing it with 5 µg of purified recombinant cII-vHa-ras protein (soluble antigen) prior to the incubation with the immobilized antigens. We also used serum 21 as a negative control (previously shown to have no response for ras protein) and serum C138 (rabbit polyclonal) as positive control in the system. The experiment indicated that antibodies against ras were compited in serum 36 and in the positive control C138 by ras fusion protein. In addition, we performed a similar competition experiment using an immuno-purified cellular ras protein as immobilized antigen. The ras protein was purified by immunoprecipitation from a mouse cell line that overexpress the c-Ha-ras protein (F33), subjected to SDS-PAGE and transferred onto nitrocellulose paper. By using this complete cellular ras protein as filter bound antigen and recombinant cII-cHa-ras protein as competitor, we tested again serum 36; we also included a weakly positive serum for ras protein (serum 20) as well as a negative one (serum 19) and normal controls (N1, N2). The results indicate that sera 36 and 20 contain specific antibodies against cellular p21ras protein, and that they were compited in the presence of purified ras protein. These results taken together indicate that about 20% of the patients presenting premalignant lesions (25% of CIN patients) contain antibodies against cellular p21ras protein.

Results Unforeseen in the Original Project: The study of cellular genes differentially regulated in human keratinocytes (such as the involucrin gene) complements the results obtained with the HPV-18 early promoter region.

Work plan and time schedule (originally envisaged)

We wish to continue with the epidemiological studies in Monterrey, and Mexico City and we will initiate similar studies in Brazil and Chile. These studies will involve the parallel determination of HPV types and also of antibodies against early HPV proteins (in particular, against the oncogenic E7 protein). It will be interesting to study if a new type of HPV or the benign (HPV-6 or HPV-11) are involved in latinamerican uterine-cervix carcinomas. In as many samples as possible we will determine ras and myc alterations (amplification and rearrangement) and over expression of oncogenic proteins. We plan to study these alterations in premalignant lesions and we will determine if HPV-16 is integrated near or inside the c-myc gene. We are also planning the study of the factors controlling transcription elongation both in c-myc and HPV-16 and HPV-18.

Our work plan also pretends the continuation of the projects on HPV gene expression, such as the mechanism of E2 down regulation of HPV transcription or the basis for the epitheliotropy of HPVs (involving specific epithelial protein factors and the AGCCAAA sequence).

Schedule

First year: We plan to consolidate our collaboration with Brazil and Chile: train a couple of students on modern techniques in molecular oncology and genetic engineering. It is our intention to complete the development of riboprobes for HPV detection and the purification of viral antigens (E4, E6, E7).

Second year: A great number of samples will be analyzed both from patients with cancer and with precancerous lesions; this is going to give an idea of the HPV types involved in mexican lesions and a possible correlation between the patient's production of antibodies against viral early proteins and the development of uterine cervix carcinoma.

Third year: A model for E2 protein and the action of epithelial specific factors will be postulated. The involvement of cellular oncogenes (myc, ras) and HPV types in uterine cervix carcinoma for latinamerican samples will be clarified. A better understanding of the imune system of the host will be obtained. This information might help in the design of a vaccine for the latin population.

Work plan and time schedule
(actual)

Project landmarks, duration of individual tasks (use bar charts); evaluation criteria (publications, patents, services, training)

We continued with epidemiological studies employing modern technology (PCR). A landmark has been the clear and solid data about the percentage of clinically normal women and of women with invasive cervical cancer presenting high-risk HPV sequences. In addition, a surprising result was obtained with cell lines derived from uterine-cervix carcinomas: all of them (8/8) presented HPV DNA and the majority contained HPV-18 sequences.

With regard to cellular oncogenes, we confirmed and expanded previous observations about c-myc involvement in the development of uterine-cervical cancer. We detected c-myc alterations in premalignant lesions and the frequency of these alterations increased with tumor invasion. These carcinomas were highly heterogeneous regarding Myc and pRb expression (as determined by in situ hybridization and immunohistochemistry). Surprisingly a frequent inverse relationship between expression levels for these genes was observed in certain areas of the tumors.

A major contribution to understand both the viral life cycle and the viral-host relationship represented the studies related to pRb and p53 inhibition of high-risk HPV early gene expression. Our results on the in vivo repression of E6/E7 viral oncogenes by the E2 protein were also of great interest to determine the HPV contribution to initiation and advancement of premalignant cervical lesions. We are now characterizing the interaction between TFIID, E2 and other transcriptional factors on the viral early promoter.

The parallel determination of HPV types and antibodies against early viral proteins (in particular, against the oncogenic E7 protein) and cellular oncogenic proteins was interesting because we detected a higher prevalence of antibodies against E7 and ras in malignant and premalignant lesions, respectively, compared to healthy individuals. These studies were in great part related to the work plan originally envisaged and could contribute not only to a better understanding of cervical cancer molecular mechanisms but to the development of diagnostic and preventive strategies. Several students both national and international (see networking) were trained in different techniques related to molecular oncology.

Schedule:

First year: We trained technitians and students from Brazil, Cuba and different cities in Mexico on modern techniques in molecular biology genetic engineering and molecular oncology. Riboprobes were constructed and samples were obtained. The technology was improved (in situ hybridization, PCR, immunohistochemistry).

Second year: Samples from patients with uterine-cervix cancer and precancerous lesions were analyzed. The presence of different HPV types together with antibodies

Work plan and time schedule
(actual)

Project landmarks, duration of individual tasks (use bar charts); evaluation criteria (publications, patents, services, training)

against viral early proteins gave us a better understanding of the patient's immunoresponse.

Third year: The molecular mechanism behind the transcriptional repression of E6 and E7 expression by the viral E2 protein was determined in vivo. We showed that the same E2 protein can function in vivo as both an activator or a repressor of transcription, depending on the distance between E2BS and the TATA box.

In order to understand the involvement of epithelial specific factors on gene regulation, we have been employing the HPV LCR and the involucrin gene promoter. The HPV early genes and the involucrin gene are similarly regulated during keratinocyte differentiation.

We have continued gaining a better understanding of the immune system in HPV infected patients, and in collaboration with Dr. Lutz Gissmann we are working in the design of a vaccine.

NETWORKING

Our group has been assimilating new technology in molecular biology and genetic engineering from different laboratories (P. Chambon, Strasbourg; M. Dahmus, U. C. Davis; M. Yaniv, Pasteur Institute; J. Di Paolo, NIH; E. Garrido, Princeton; T. Benjamin, Harvard; L. Gissmann, Heidelberg, and Loyola Univ. Chicago). We developed the technology for in situ hibridization (for both RNA and DNA), immunohistochemistry, PCR, RT-PCR-sequencing, etc. We are now in the process of transferring it to different Institutions: a) in Mexico (National Institute of Public Health, Cuernavaca; National Cancer Institute; National Medical Center; UNAM; UANL, Monterrey; UAEM, Toluca, etc); (b) in other countries (INOR, Cuba; National Cancer Institute, Rio de Janeiro, Brazil, etc). We have trained students from Hospitals, Clinics and Universities in Mexico. In addition, our group trained Madelyn Ramírez from INOR, Cuba and Mónica Pereira from the National Cancer Institute, Rio de Janeiro.

PUBLICATIONS

1. Gariglio P. Genética molecular del cáncer. Inf. Cient. y Tecnol. (CONACYT), 15 • 201, (1993):24-27.

2. Garcia A, y Gariglio P. Aspectos moleculares de los papilomavirus humanos y su relación con el cáncer cérvico-uterino. Rev. Invest. Clin. 45(1993) 85-92.

3. Rangel LM, Ramírez M, Torroella M, Pedroza A, Ibarra V, Gariglio P. Multistep carcinogenesis and Genital Papillomavirus Infection. Implications for Diagnosis and Vaccines. Archives of Medical Research (1994) 25:2, 265-272.

4. Gariglio P, and Salcedo M. Molecular genetics of uterine cervix carcinoma. Involvement of c-myc oncogene. XVII World Congress of Anatomic and Clinical Pathology. Editor G. Santoscoy. MONDUZZI Editore (Italy) (1994) 295-300.

5. Alvarez, L.,Velázquez, A., López, E., Woodworth, C. D., Garrido, E., Gariglio, P. and Dipaolo, J. A. Transcriptional repression in normal human keratinocytes by wild-type and mutant p53. Cancer Letters (1995) 91:85-92.

6. Lopez, E., Vega A., Cadena A., Granados S., Jave L., Gariglio P., and Alvarez, L. Transcriptional analysis of the 5′ non-coding region of the human involucrin gene. J. Biol. Chem. (In press).

7. Salcedo, M.,Garrido, E., Taja, L,. and Gariglio, P. The Retinoblastoma Gene product Negatively Regulates Cellular or Viral Oncogene Promoter **in vivo**. Submitted to Archives Med. Res. August 1995.

8. Garrido, E., Carrillo, E., and Gariglio, P. In vivo regulation of the early promoter of HPV-18 by BPV1 E2 protein is dependent on the distance between the proximal E2 binding site and the TATA box. Submitted to J. Virol. 1995.

Part 4

STATEMENT OF EXPENDITURES

To be filled by ICGEB		To be filled by the Affiliated Centre	
Budgets as per original proposal		**Summary of expenditures** *	
1) Capital equipment	US$	1) Capital equipment	US$ 11,366.09
2) consumables	US$	2) consumables	US$ 28,633.91
3) training	US$	3) training	US$ .....
4) literature	US$	4) literature	US$ .....
5) miscellaneous	US$	5) miscellaneous	US$ .....
TOTAL GRANT	**US$**.....	**TOTAL**	**US$** 40,000.00

Please itemize the following budget categories (if applicable)

Capital equipment

-Gene pulser aparatus
-Standard microscope
-Termoblock
-Leading Edge Computer
-Freezer

Training (provide names, duration of training, host laboratory)

1.- Cadena C. Adriana (19912-1993)
2.- Guido J. Miriam (1992-1993)
3.- Aguilar L. Adriana (1993-1994)
4.- Castañeda P. Cristina (1993-1994)
5.- Moralez Nestros (1992-1994)
6.- Carillo Elva (1993-1994)

Literature

P.Gariglio, M.Salcedo. Molecular genetics of uterine cervix carinoma. Involvement of c-myc oncogene. XVII World congress of Anatomic and Clinical Pathology. Acapulco, México. pp 295-300. 1993.

L.M.Rangel, M.Ramírez, M.Torroella, A.Pedroza, V.Ibarra, and P.Gariglio. "Multistep Carcinogenesis and genital Papillomavirus Infection. Implications for Diagnosis and Vaccines. Arch.Med.Res. 25(2):265-272. 1994.

Garrido E., Carrillo E., and Gariglio P. In vivo regulation of the early promoter of HPV-18 by BPV1 E2 protein is dependent on the distance between the proximal E2 binding site and the TATA box. Submitted to J. Virol 1995.

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