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c17-ORF37 AND HER2 RELATIONSHIP: MOLECULAR AND FUNCTIONAL STUDIES

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1. INTRODUCTION

Cancer is the leading killer in the United States, surpassing heart disease in 2004. According to the American Cancer Society, more than 1,500 people die each day from cancer and over 16 million people have been diagnosed with cancer since 1990.

In recent years, medical science has taken great strides in understanding and treating cancer. However, prior to 1997, the majority of treatments such as chemotherapy and radiation, were not based on the underlying biology of cancer growth. These treatments are also often associated with serious side effects.

Cancer is the result of oncogenic transformation of normal cells to produce cells which have the potential for uncontrolled division, producing a tumour cell mass which invades and destroys the tissue in which it arose, and has the potential to metastatize. It is now recognized that oncogenic transformation is the result of genetic mutations affecting systems controlling the cell cycle and cell death. This has implications for the way in which anti-cancer therapies are developed, because the identification of factors or pathways that are altered in cancer cells may allow the development of specific therapies that target these changes (Hortobagyi, 1999).

Growth factors act by binding to cell surface receptors and activating pathways that result in the expression or inhibition of proteins that control cell function. In contrast, tumour suppressor genes are involved in the control of programmed cell death (apoptosis) and prevent uncontrolled proliferation. The balance between the expression of proto-oncogenes and tumour suppressor genes is critical if tissues have to grow and be maintained normally. Research has shown that a series of mutations in these genes is at least partially responsible for the progression from normal breast epithelium to breast cancer and the maintenance of the malignant phenotype (Aaronson, 1991, Harris, 1992). Genes that have been implicated in the pathogenesis of breast cancer include c-myc (Escot, 1986), H-ras (Agnantis, 1986), hst (Lidereau, 1988), int2 (Lidereau, 1988), p53 (Cossman & Schlegel, 1991, Kovach, 1991) and the human epidermal growth factor receptor (HER) gene family (Benz ,1992, Chazin, 1992).

1.1. THE HER FAMILY

The HER family comprises four genes encoding four homologous ErbB or type I tyrosine kinase receptors: epidermal growth factor receptor (ErbB1, EGFr or HER1), ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4) (Pinkas- Kramarski, 1997). The various naming systems for these genes are the result of the discovery of homologous genes involved in the pathogenesis of erythroblastoma in chickens (erbB) (Vennstrom and Bishop, 1982) and of genes encoding growth factor receptors in humans (HER). However, HER is the most commonly used designation.

The HER receptors are located at the cell membrane and share similar structure, comprising an extracellular growth factor binding domain, a lipophilic transmembrane domain and an intracellular protein kinase domain with a regulatory C-terminal segment (van der Geer, 1994).

A physical feature of the HER pathway is that the signaling system always involves two receptors in combination (dimer): homodimers are combinations of two similar receptor types, heterodimers contain two different receptors The various hetero- and homodimer pairs affect the signal strength within the cell. For example, the co-expression of certain pairs (such as HER3/HER2) is more powerful than others such as the HER3/HER-3 homodimer, which is inactive.

1.1.1. STRUCTURE AND FUNCTION OF HER2

The HER2 gene is located on the long arm of chromosome 17 (Coussens, 1985). Normal cells contain two copies of the HER2 gene, which encodes a protein comprising 1255 amino acids of molecular weight 185 kDa (Akiyama, 1986). This protein has homology to, and conforms with, the general structure of the HER proteins (Coussens, 1985, Hynes & Stern, 1994).

HER2 and the three other members of the HER family are transmembrane tyrosine kinase receptors with growth-stimulating activity. Tyrosine kinases are enzymes that transfer phospho-groups from adenosine triphosphate to tyrosine molecules in proteins, resulting in their activation. Activation of HER receptors ultimately results in nuclear gene activation.

1.1.2. HER2 ACTIVATION

HER2 activation is usually dependent on the presence of small molecules called ligands (Akiyama, 1988, Kokai, 1989) and other HER receptors (Connelly & Stern, 1990). The ligands involved in HER2 activation include epidermal growth factor (Akiyama, 1988) and neuregulins (Plowman, 1993), which interact specifically with HER receptors. However, it appears that these ligands can activate HER2 only when it is associated with HER1, HER3 or HER4 in the form of a heterodimer (Alroy & Yarden, 1997, Lemmon & Schlessinger, 1994). Infact, while no known specific ligand for the HER-2 receptor has been identified, evidence indicates that HER2 is the preferred heterodimerization partner within the HER family (Graus-Porta, 1997) and that HER2-containing heterodimers have particularly high ligand-binding potency (Sliwkowski, 1994). Furthermore, the effect of the binding of ligands to HER heterodimers varies depending on the specific molecules involved (Hynes & Stern, 1994), i.e. the effect of epidermal growth factor binding to a HER1-HER2 heterodimer may differ from that of neuregulin binding to a HER2-HER3 heterodimer. Therefore, the interactions between HER2 and the other HER proteins and the activity of the various known ligands are important to the complex processes controlling cell growth and differentiation.

1.1.3. NORMAL BIOLOGICAL FUNCTION OF HER2

HER2 is expressed in many cell types outside the haematological system, including nervous system, bone, muscle, skin, heart, lungs and intestinal epithelium (Coussens, 1985). Expression levels are higher in foetal tissues than adult tissues (Press, 1990). The activity of the HER2 protein in these tissues is affected by which ligands and other HER receptors are present. For example, HER1 and HER2 are often co-expressed and neuregulins and EGF-like ligands are known to be able to activate HER2 in an autocrine and paracrine fashion (Marchionni, 1993). The activity of HER2 has been most widely studied in breast cancer cell lines. Studies have demonstrated that EGF-like ligands and neuregulins can inhibit or stimulate cell proliferation depending on the cell line tested (Peles, 1992, Lewis, 1993 a)), with

some growth-inhibited cells having the characteristics of differentiated breast epithelial cells (Peles, 1992).

1.2. ROLE OF HER2 IN CANCER

The role of the HER receptors in controlling cell growth and the importance of HER2 as a heterodimerization partner suggest that HER2 may have a critical role in the development of cancer.

1.2.1. HER2 AMPLIFICATION/ OVEREXPRESSION IN HUMAN TUMOURS

Studies of human cancers have demonstrated that HER2 gene amplification (on 17q12 chromosome) and/or HER2 receptor overexpression are observed in a variety of tumour types. Such tumour types include breast (Slamon, 1989, Berns, 1992), ovarian (Slamon, 1989, Hynes & Stern, 1994), pancreatic (Lei, 1995) and lung cancer (Weiner, 1990, Kim, 1998). The data from studies of cell lines and animal xenograft models suggest that these findings are not incidental, i.e. HER2 amplification/overexpression is involved in malignant transformation rather than being a marker of cancer cells that has no role in their production.

HER2 amplification/ overexpression has been detected in varying proportions of a range of human cancers. The percentage of tumours in which the HER2 gene is amplified is usually lower than the percentage in which the protein is overexpressed. The incidence of HER2 gene amplification is 25±30% for breast and 15±30% for ovarian carcinomas, with the incidence of HER2 protein overexpression being slightly higher (Slamon, 1987, 1989).

In vitro studies of HER2 overexpression in breast cancer cells indicate that HER2 gene amplification occurs in 92% of breast cancer specimens overexpressing the HER2 receptor (Pauletti, 1996). The role of HER2 receptor overexpression in the absence of gene amplification is unknown. It also appears that gene amplification is distinct from aneuploidy or the presence of multiple copies of the normal chromosome complement of a cell: breast cancers containing aneuploid cells with multiple copies of chromosome 17 behave similarly to cancers with normal HER2 gene copy numbers.

It has been demonstrated that HER2 gene amplification is an early event in the development of cancer (Chong, 1999). In this study, sections of pure ductal

carcinoma in situ (DCIS), DCIS associated with invasion and invasive tumours were analysed using a sensitive radiolabelling technique. This revealed that the frequency distribution of HER2 expression was similar in DCIS and DCIS with invasion and in DCIS with invasion and invasive cancer. Furthermore, HER2 expression levels were similar in the DCIS and invasive components of DCIS with invasion. Others have shown that HER2 amplification/overexpression is strongly associated with the poorly differentiated type of DCIS, which is more likely to progress to invasive breast cancer (Barnes, 1992, Liu, 1992).

1.2.3. HER2 AND ONCOGENIC TRANSFORMATION

As stated above, HER2 amplification/overexpression is associated with DCIS and invasive breast cancer. However, HER2 amplification/ overexpression is not observed in normal breast cells, proliferative disease without atypia and proliferative disease with atypia. Therefore, HER2 amplification and cell transformation occur at approximately the same time. However, it appears that oncogenic transformation usually begins with an unknown initial event. If breast cancer cells are transfected with the HER2 gene, they become fully transformed and exhibit aggressive growth characteristics (Benz, 1992, Chazin, 1992). In contrast, normal breast cells are not affected by HER2 gene transfection. This indicates that oncogenic transformation is at least a two-stage process involving an initiating event followed by HER2 gene amplification, which occurs for as yet unknown reasons.

HER2 gene amplification results in epidermal cells containing more than the normal two gene copies. For practical purposes, five gene copies per cell may be considered as an appropriate cut-off point for defining gene amplification (Liu, 1992). When the HER2 gene is amplified, HER2 mRNA levels and ultimately HER2 protein levels are increased. HER2 protein is then overexpressed on the cell surface at levels 10- to 100-fold greater than normal (Venter, 1987). This appears to result in the formation of HER2 homodimers, which are probably active without the need for ligand binding. This abnormal activity results in unregulated cell growth, in some instances resulting in cancer (Di Fiore,1987, Hudziak, 1987).

The overexpressed HER2 gene product is usually normal (Pauletti, 1996), i.e. it is

identical to the HER2 receptor found in normal cells. No mutational changes in the HER2 protein in human cancers have been reported (Aaronson, 1991).

Thus, in the majority of cases the presence of increased numbers of the HER2 gene results in overexpression of structurally normal HER2 protein (Hynes & Stern, 1994). It is possible that abnormalities of the control of HER2 mRNA formation or translation to produce protein are the cause of HER2 mRNA or receptor overexpression in tumours in which HER2 gene amplification is not present (Slamon, 1989).

1.3. HERCEPTIN™ THERAPY

1.3.1. DEVELOPMENT OF HERCEPTIN™

Murine monoclonal antibodies (muMAbs) can be generated to target a specific antigenic determinant on a protein. muMAbs directed against the extracellular domain of the HER2 receptor inhibit proliferation of monolayer cultures of breast and ovarian tumour cells overexpressing HER2 (Hudziak, 1989, McKenzie, 1989).

The antibody designated muMAb 4D5 was found to be particularly potent and was selected for further clinical development.

In clinical practice muMAbs have limited use because the human immune system recognizes murine antibodies as foreign and develops an immunogenic response to them (Miller, 1983).

Using recombinant technologies, trastuzumab (Herceptin™), a monoclonal IgG1 class-humanized murine antibody, was developed by Genentech (South San Francisco, CA) to specifically bind the extracellular portion of HER2. Herceptin™ is 95% human and 5% murine and demonstrates a higher binding affinity for the extracellular domain of HER2 than muMAb 4D5 (Lewis, 1993 b)).

This antibody therapy was initially targeted specifically for patients with advanced relapsed breast cancer that overexpressed the HER2/neu protein (Huston, 2001). Since its launch in 1998, trastuzumab has become an important therapeutic option for patients with HER2-positive breast cancer.

The addition of trastuzumab to chemotherapy (either anthracycline plus cyclophosphamide or taxane) was associated with a longer time to disease progression, a higher rate of objective response, a longer duration of response, a lower rate of death at 1 year, longer survival, and a 20% reduction in the risk of death (Slamon, 2001). Although not completely understood at this time, mechanisms believed to be associated with the anti-tumour effects of trastuzumab include ADCC (antibody depended cytotoxicity), immune system mediated prevention of HER2 dimerization with reduced or interrupted downstream signalling, and so on (Nahata, 2003).

1.3.2. PREDICTING RESPONSE TO THERAPY

HER-2 STATUS AND THE PREDICTION OF RESPONSE TO TRASTUZUMAB THERAPY.

The best method to identify patients who may respond to trastuzumab therapy has been a source of controversy. The original IHC technique used in the trastuzumab pivot trial was succeeded by two commercially available HER2 IHC kits, the Dako Herceptest™ and the Ventana Pathway™, both FDA-approved, but, after its approval and launch, the Herceptest™ assay was criticized for yielding false-positive results.

Concern over IHC accuracy using standard formalin-fixed paraffin-embedded tissue sections has encouraged the use of the FISH (Fluorescence in Situ Hybridazation) assay for its ability to predict trastuzumab response rates. Two versions of the FISH assay are FDA-approved; the Ventana Inform™ test that measures only HER2 gene copies and the Abbott-Vysis Pathvysion™ test that includes a chromosome 17 probe in a dual colour format. In a recently published study where trastuzumab was used as a single agent, the response rates in patients with 3+ IHC staining was 35% and the response rates for 2+ cases was 0%; the response rates in patients with and without HER-2/*neu* gene amplification detected by FISH were 34 and 7%, respectively (Vogel, 2002). Similar results were obtained in another study of breast cancer treated with trastuzumab plus paclitaxel, in patients with HER2 overexpressing tumours.

Other recent studies have favoured the FISH approach not only to confirm 2+ IHC cases but to also confirm 3+ and prevent the use of potentially toxic trastuzumab to patients with false-positive IHC results who are unlikely to benefit from this therapy (Hammock, 2003).

So it can be concluded that gene amplification plays an important role in the progression and initiation of breast cancer, as amplification and consequent overexpression of HER2 oncogene is found in 10-34% of primary human breast tumours and is associated with poor clinical outcome (Ross, 1998). So Herceptin™ has been successfully used for treatment of patients with advanced breast cancer, but not all patients with HER2 receptor overexpression, if not associated with gene amplification, respond favourable to Herceptin™ therapy.

1.4. HER2 AMPLICON ON HUMAN CHROMOSOME 17q12

These recent discoveries have directed further interest toward HER2 oncogene, especially to the identification of additional genetic factors that might influence the treatment responses of HER2-positive tumours. Several genes, located adjacent to HER2, have been shown to be frequently amplified and overexpressed together with HER2 indicating that the 17q12 amplification leads to simultaneous activation of multiple genes. These co-amplified and overexpressed genes might be likely candidates for factors that have an impact on the treatment responses observed in the HER2-amplified tumours (Kauraniemi, 2003).

It has been performed a detailed characterization of the structure of the HER2 amplicon in breast cancer cell and it has been defined a minimal common region of amplification at 17q12, restricted to a less than 0.5-Mb region around the HER2 locus (Kauraniemi, 2001). They next showed that expression levels of most of the genes located in this minimal common region of amplification were elevated. Although several studies have illustrated that genetic aberrations observed in cell-line model systems are highly representative of those occurring in primary tumours, it is essential to confirm results obtained from such model systems in primary tumours. Such confirmation is especially relevant in the case of amplicons because previous studies have suggested that the size of amplicons decreases as a function of time. The initial amplicons are thought to be large but during subsequent cell cycles, progressively smaller regions are selected for leading to a reduction in the amplicon size (Stark , 1989).

The 17q12 amplicon, as shown in a large set of primary breast tumours, has a core region of amplification spanning only 280 kb. The minimal common region of amplification at 17q12 contains a total of 10 transcripts (ERBB2, GRB7, MLN64, PNMT, NEUROD2, ZNFN1A3, TCAP, PPP1R1B and two hypothetical proteins MGC14832 and MGC9753), including the HER2 oncogene. Quantitative real-time RT-PCR analysis revealed that only six of these transcripts showed a strong statistically significant correlation between amplification and expression levels indicating that increased gene copy number does not inevitably lead to elevated expression. In addition to HER2, expression levels of five other genes, GRB7,

MLN64, PNMT, MGC9753, and MGC14832, were consistently elevated in tumours with amplification (Kauraniemi, 2003).

The fact that amplification leads to elevated expression of several but not all genes within an amplicon argues against the traditional concept in which there is a single target gene for each amplicon. However, it cannot be directly assumed that all of these genes with elevated expression levels would have important and independent roles in tumorigenesis. It is indeed possible that increased expression of a particular gene represents a simple by-product of co-amplification and does not confer any advantage for the cancer cell. However, it is also possible that the coordinated effect of deregulated expression of several genes is essential for the selective growth advantage of the tumour cells.

The GRB7 and MLN64 genes have been previously shown to be co-amplified and overexpressed with HER2 (Tomasetto, 1995) and, based on their function, increased expression of these genes might be important for cancer pathogenesis.

GRB7 codes for a SH2 domain-containing growth factor receptor tyrosine kinase that has been shown to function in cell migration, thus suggesting a possible role for GRB7 in metastasis.

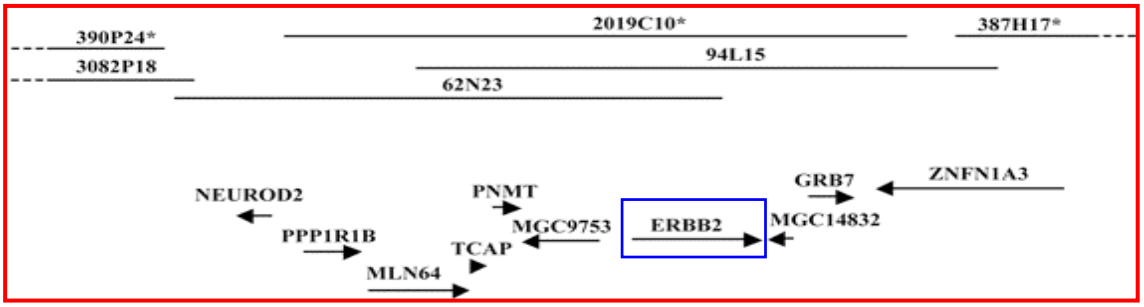
MLN64 shares significant homology with the steroidogenic acute regulatory protein and it has been proposed to facilitate steroid hormone production in cancer cells.

Although statistically associated with gene amplification, the expression levels of PNMT were rather low and not consistently elevated in the amplified tumour group, therefore decreasing its relevance as a putative cancer gene.

On the contrary, the hypothetical proteins MGC9753 and MGC14832 showed highly significant association between gene amplification and expression levels.

Unfortunately, their sequence shows no similarity to any currently known genes or proteins, therefore leaving their function and possible role in cancer unresolved.

The potential contribution of two hypothetical proteins, MGC9753 and MGC14832, to the development and progression of breast cancer or to the clinical behaviour of HER2-amplified tumours needs to be evaluated.



Physical map of the 17q12-q21 region. Schematic representation of the 280-kb minimal region of amplification located between clones 390P24 and 387H17. Transcripts mapping to the minimal region are represented with arrows and their orientation is indicated. The maps were not drawn in scale.

1.5. HER2 AMPLICON ON MOUSE CHROMOSOME 11.

Mouse *Ppp1r1b, Stard3, Tcap, Pnmt, Mgc9753, Erbb2, Mgc14832*, and *Grb7* genes were located around the synthenic locus on mouse chromosome 11, corresponding to the PPP1R1B-GRB7 locus on human chromosome 17q12. Mouse *Ppp1r1b-Grb7* locus and human PPP1R1B-GRB7 locus were evolutionarily conserved in the order and the orientation of genes therein (Katoh & Katoh, 2003).

A

Mouse chromosome 11 (NT_039521.1)

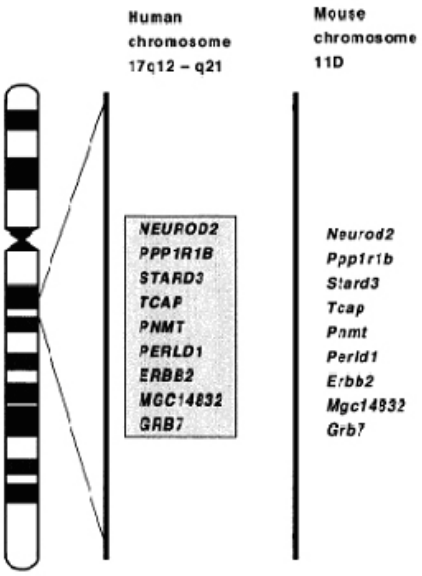
B

Gene symbol	Aliases	Protein function	Substitution rate between mouse and human (%)	
			Nucleotide	Amino acid
Neurod2	*Ndrf*	bHLH transcription factor	7.8	2.4
Ppp1r1b	*Darpp32*	Phosphatase inhibitor subunit	17.8	10.8
Stard3	*Mln64*	START domain protein	11.7	6.5
Tcap	*Telethonin*	Titin-cap sarcomeric protein	14.5	10.2
Pnmt	*Pent*	Phenylethanolamine N-methyltransferase	17.0	14.9
Mgc9753		Seven – transmembrane receptor	14.3	15.0
Erbb2	*Neu, Her2*	Receptor tyrosine kinase	14.5	12.5
Mgc14832	*1810046J19Rik*	Uncharacterized	11.2	9.6
Grb7		RalGDS – PH – SH2 domain protein	14.0	9.4
Znfn1a3	*Aiolos*	Zinc finger protein	14.7	14.3

Comparative genomics on the *PPP1R1B-GRB7* locus. **A**, schematic presentation around the **mouse *Ppp1r1b-Grb7* locus**. Mouse genome around the *Ppp1r1b-Grb7* locus is shown by bold horizontal bar, while mouse genes are shown by an arrow with gene symbol. Mouse *Ppp1r1b-Grb7* locus and human *PPP1R1B-GRB7* locus were evolutionarily conserved in the order and the orientation of genes therein. **B**, comparative genomics on the *Ppp1r1b-Grb7* locus. Gene symbol, alias, and function of genes around the *Ppp1r1b-Grb7* locus are shown. Coding region nucleotide substitution rate and amino-acid substitution rate between human and mouse orthologs around the *Ppp1r1b-Grb7* locus are also shown

Mouse *Erbb2* gene, consisting of 27 exons, was located, in the forward orientation, between the mouse *Mgc9753* gene, in the reverse orientation, and mouse *Mgc14832*

gene, in the reverse orientation too, within the *Ppp1r1b-Grb7* locus on the mouse chromosome 11. Mouse Erbb2 protein (1256 aa) showed 87.5 and 95.2% total amino-acid identities with human ERBB2 and rat Erbb2 proteins, respectively.



Comparative genomics of the oncogenomic recombination hotspot around the *PPP1R1B-PERLD1-ERBB2-GRB7* amplicon (grey box) on human chromosome 17q12-q21 and mouse chromosome 11D.

1.6. OXIDATIVE STRESS, ANTIOXIDANT DEFENSES, AND DAMAGE REMOVAL, REPAIR, AND REPLACEMENT SYSTEMS

Oxidative stress is an unavoidable consequence of life in an oxygen-rich atmosphere. Oxygen radicals and other activated oxygen species are generated as by-products of aerobic metabolism and exposure to various natural and synthetic toxicants. The “Oxygen Paradox” is that oxygen is dangerous to the very life-forms for which it has become an essential component of energy production (Davies, 2000).

The 1st defence against oxygen toxicity is the sharp gradient of oxygen tension, seen in all mammals, from the environmental level of 20% to a tissue concentration of only 3–4% oxygen. These relatively low tissue levels of oxygen prevent most oxidative damage from ever occurring. Cells, tissues, organs, and organisms utilize multiple layers of antioxidant defences and damage removal, and replacement or repair systems in order to cope with the remaining stress and damage that oxygen engenders. The enzymes comprising many of these protective systems are inducible under conditions of oxidative stress adaptation, in which the expression of over 40 mammalian genes is upregulated. Mitotic cells have the additional defensive ability of entering a transient growth-arrested state (in the 1st stage of adaptation) in which DNA is protected by histone proteins, energy is conserved by diminished expression of nonessential genes, and the expression of shock and stress proteins is greatly increased. Failure to fully cope with an oxidative stress can switch mitotic cells into a permanent growth-arrested, senescence-like state in which they may survive for long periods. Faced with even more severe oxidative stress, or the declining protective enzymes and adaptive capacity associated with aging, cells may “sacrifice themselves” by apoptosis, which protects surrounding healthy tissue from further damage. Only under the most severe oxidative stress conditions will cells undergo a necrotic death, which exposes surrounding tissues to the further vicissitudes of an inflammatory immune response. This remarkable array of systems for defence, damage removal, replacement, repair, adaptation, growth modulation and apoptosis make it possible for us to enjoy life in an oxygen-rich environment.

Thankfully, we are not defenceless against the oxygen radical and other activated-oxygen species to which we are constantly exposed. All aerobic organisms, including human beings, utilize a series of primary antioxidant defences in an attempt to protect against oxidant damage.

1.6.1. ANTIOXIDANT COMPOUNDS

Our cells utilize a series of antioxidant compounds to directly react with oxidizing agents and disarm them (Ozben, 1998); such antioxidants are said to be "scavengers". Vitamin E (α-tocopherol) is a major membrane-bound antioxidant, and vitamin C (ascorbic acid) is a major aqueous-phase antioxidant. Other water-soluble antioxidant compounds include uric acid, glutathione, and ceruloplasmin; by binding iron and/or copper, uric acid may inhibit metal-catalyzed oxidation reactions without itself becoming oxidized. Other lipid-soluble agents, including β-carotene and ubiquinone, may also play important antioxidant roles in vivo.

1.6.2. ANTIOXIDANT ENZYMES

Aerobic organisms also synthesize numerous antioxidant enzymes and other proteins in an attempt to minimize oxidative damage (Fridovich, 1995); the best known of these enzymes is superoxide dismutase. The superoxide dismutases (SOD) catalyze the reaction $O_2^{\cdot -} + O_2^{\cdot -} \rightarrow H_2O_2 + O_2$. Genetic deletion of SOD has been shown to be a lethal mutation in lower organisms, underpinning the essential importance of this enzyme family. The product of SOD is H_2O_2, which is clearly toxic and must be rapidly removed. In mammalian cells this is accomplished by two enzyme families: the glutathione peroxidases and the catalases. Both glutathione peroxidases and catalases detoxify H_2O_2 by reducing it to water and oxygen.

1.6.3. TRANSIENT GROWTH ARREST

One of the surprises to emerge from studies of cellular stress responses is the observation that transient growth-arrest is utilized by mitotic cells to protect against

acute stress (Wiese, 1995). Our current understanding or interpretation is that the DNA of dividing cells is much more susceptible to damage than is that of postmitotic cells. In postmitotic cells, DNA is, for the most part, maintained in a supercoiled state and is further protected by a coating of histone proteins. In contrast, the DNA of mitotic cells spends long periods of time in an uncoiled state, with no histone protein coating, in order to undergo replication. This uncoiled and naked state of DNA in dividing cells makes them especially susceptible to various forms of damage, including oxidative stress. The situation is further exacerbated by the heavy metabolic demands of very high rates of transcription, translation, and the multiple processes leading to division in mitotic cells. Thus, an early response of mitotic cells to oxidative stress is to enter a transient growth-arrested state in which DNA is largely supercoiled, replication is halted, and only a few shock or stress genes are transcribed and translated (Wiese, 1995). If the oxidative stress is not severe enough to cause apoptosis or necrosis, mammalian cells will re-enter the growth cycle after some 3 to 4 h of transient growth-arrest, undergo adaptive changes, and actually exhibit increased tolerance to oxidative stress after several hours.

1.6.4. REPAIR SYSTEMS

Damage repair systems may be classified as either direct or indirect (Davies, 1995). Direct repair, about which we know only a little, has so far only been demonstrated for a few classes of oxidized molecules. Some important direct repair processes are the re-reduction of oxidized sulfhydryl groups on proteins, the direct repair of DNA hydroperoxides by glutathione peroxidase.

Extensive studies have revealed that oxidized proteins are recognized by proteases and completely degraded (to amino acids); entirely new replacement protein molecules are then synthesized de novo (Sitte, 2000). It appears that oxidized amino acids within oxidatively modified proteins are eliminated or used as carbon sources for ATP synthesis. Because an oxidatively modified protein may contain only two or three oxidized amino acids, it appears probable that most of the amino acids from an oxidized and degraded protein are reutilised for protein synthesis. Thus, during oxidative stress many proteins synthesized as damage replacements are likely to

contain a high percentage of recycled amino acids. During periods of particularly high oxidative stress, the proteolytic capacity of cells may not be sufficient to cope with the number of oxidized protein molecules being generated.

Under such circumstances, oxidized protein may not undergo appropriate proteolytic digestion and may instead cross-link with one another or form extensive hydrophobic bonds. Such aggregates of damaged proteins are detrimental to normal cell functions and lead to further problems.

In bacteria such as *Escherichia coli*, a series of proteolytic enzymes act cooperatively in the recognition and degradation of oxidatively modified soluble proteins. A similar series of proteolytic enzymes appear to conduct the degradation of oxidatively modified soluble proteins in mammalian mitochondria. In the cytoplasm and nucleus of eukaryotic cells, however, oxidized soluble proteins largely appear to be recognized and degraded by the proteasome complex, which constitutes up to 1% of total cellular protein.

Lipid peroxidation was the 1st type of oxidative damage to be studied. Membrane phospholipids are continually subjected to oxidant challenges. Peroxidized membranes become rigid, lose selective permeability, and under extreme conditions, can lose their integrity. Peroxidized membranes and lipid oxidation products represent a constant threat to aerobic cells. It is now widely held that in addition to preventing initiation of peroxidation (with compounds such as vitamin E), cells have also developed a variety of mechanisms for maintaining membrane integrity and homeostasis by repairing oxidatively damaged lipid components.

Ribo- and deoxyribonucleic acids are also vulnerable to oxidative damage, and perhaps most importantly, DNA has been shown to incur oxidative damage in vivo (Davies, 1995). Although DNA is a relatively simple biopolymer made up of only four different nucleic acids, its integrity is vital to cell division and survival. Oxidative alterations to nucleic acid polymers has been shown to disrupt transcription, translation, and DNA replication, and to give rise to mutations.

Several eukaryotic glycosylases that act on DNA oxidation products have been characterized. A 3'-repair diesterase in yeast is apparently responsible for removing damaged 3' termini left by free radical reactions. A mammalian endonuclease has been isolated based on its specificity for oxidatively modified DNA.

There is substantial evidence for the vital role of redoxyendonucleases in higher eucaryotic cells. DNA damage that appears in cells as a result of an acute oxidant challenge (including base damage and single-strand breaks) has been shown to diminish as a function of time. These results suggest that a removal of lesions is being carried out by intracellular systems. Oxidatively damaged bases (8-hydroxydeoxyguanosine , thymine glycol, and thymidine glycol) have been measured in animal urine.

1.6.5. APOPTOSIS PROTECTS SURROUNDING CELLS

A fraction of cells exposed to high levels of oxidative stress will enter the apoptotic pathway. The mechanism of oxidative stress-induced apoptosis appears to involve loss of mitochondrial transmembrane potential (Zamzani, 1997), release of cytochrome *c* to the cytoplasm (Reed, 1997), loss of bcl-2 (Kane, 1993), downregulation and degradation of mitochondrially encoded mRNA, rRNA, and DNA (Crawford, 1998), and diminished transcription of the mitochondrial genome (Kristal, 1994). Current thinking about toxicant-induced apoptosis suggests that, in multicellular organisms, the repair of severely damaged cells represents a major drain on available resources. To avoid this difficulty, it is suggested, individual cells within organisms (or organs or tissues) will “sacrifice” themselves for the common good of the many. Apoptotic cells are characterized by “blebbing,” nuclear condensation, and DNA laddering . Such cells are engulfed by phagocytes that prevent an immune reaction and recycle usable nutrients (Farber, 1990). Under high oxidative stress, some cells will simply disintegrate or become necrotic. Unfortunately, badly damaged cells that die by necrosis cause major immune and inflammatory responses, which may cause further damage to surrounding cells and tissues. Thus, for a tissue, organ, or organism, removing badly damaged cells by apoptosis represents a very real advantage (over necrosis) and should be considered as one of the “defense mechanisms”. Furthermore, over expression of the p53 gene has been seen to result in induction of multiple “redox-related” gene products, and initiation of apoptosis (Polyac, 1997). These observations support a strong involvement of oxidative stress mechanisms in general apoptotic pathways.

1.7. CELLULAR RESPONSE TO OXIDATIVE STRESS: SIGNALING FOR SUICIDE AND SURVIVAL

At the cellular level, oxidant injury elicits a wide spectrum of responses ranging from proliferation to growth arrest, to senescence, to cell death. Typically, low doses of ROS, particularly hydrogen peroxide, are mitogenic and promote cell proliferation, while intermediate doses result in either temporary or permanent growth arrest, such as replicative senescence. Very severe oxidative stress ultimately causes cell death via either apoptotic or necrotic mechanisms. The particular outcome observed can vary significantly from one cell type to the next, as well as with respect to the agent examined, its dosage and/or duration of treatment. However, whatever the effect seen, it largely reflects the balance between a variety of intracellular stress signaling pathways that are activated in response to the oxidative insult. These pathways exert their phenotypic influences largely through modulation of transcription factor activities that effect changes in the pattern of gene expression. Some of the pathways are clearly linked to enhanced survival, while others are associated with cell death.

1.7.1. PI3-KINASE/AKT PATHWAY

Akt, also known as protein kinase B, is a serine/threonine kinase which plays a key role in integrating cellular responses to growth factors and other extracellular signals (Kandel and Hay, 1999; Meier and Hemmings, 1999). Akt is activated in response to these signals via a phosphoinositide 3-kinase (PI3K) pathway in which PI3K-mediated generation of 3'-phosphorylated phosphoinositides leads to the recruitment of Akt to the cell membrane where it undergoes phosphorilation by kinases such as the 3'-phosphoinositide-dependent kinase-1 (PDK-1). Akt is an important anti-apoptotic protein through which survival signals suppress cell death induced by growth factor withdrawal, cell cycle discordance, and detachment of cells from their extracellular matrix.

Akt is activated in response to oxidant injury as well as several other stresses known to exert their cytotoxic effects in part through generation of ROS or perturbations in cellular redox status (Sonoda, 1999; Klotz, 2000; Wang, 2000; Huang, 2001).

Activation of Akt in response to oxidant exposure appears to be mediated largely through growth factor receptors. For example, activation of Akt by hydrogen peroxide can rely on the EGF receptor (Wang, 2000), on PDGF receptor (Klotz , 2000), or on Syk, a non-receptor protein tyrosine kinase (PTK), (Ding, 2000). Regardless of the particular receptor implicated in initiating the early events, all of the above mentioned studies demonstrated a requirement for PI3K in mediating the activation of Akt.

Evidence suggesting that activation of PI3K/Akt signaling during the cellular response to oxidant injury was important for survival was initially obtained using the PI3K inhibitor Wortmannin. Treatment of cells with Wortmannin blocked activation of Akt by hydrogen peroxide and increased cell death (Sonoda, 1999). Direct support for the importance of this pathway in enhancing cell survival following oxidant injury was subsequently obtained in studies where Akt expression and activity were modulated using a genetic approach (Wang, 2000) showing to enhance survival of hydrogen peroxide-treated HeLa and NIH3T3 cells.

The PI3K/Akt pathway is believed to transduce its survival signals through the phosphorilation-dependent suppression of intracellular apoptotic factors such as BAD, caspase 9, forkhead transcription factor, GSK3, and IKKa (Datta, 1991; Kandel and Hay, 1999). For the most part, there has been little evidence linking Akt-dependent regulation of these factors to cell survival during oxidant injury; however, many studies, not only offer a mechanism for Akt's protective influence during oxidant injury, but point to the existence of important avenues for cross talk between the PI3K/Akt and JNK signaling pathways.

1.7.2. NUCLEAR FACTOR kB (NF-kB) SIGNALING

The NF-kB family of transcription factors is composed of homodimers or heterodimers of Rel proteins that are involved in regulating a large number of genes related to immune function, inflammation, apoptosis, and cell proliferation (Pahl, 1999). Since many of the treatments known to activate the transcription factor also lead to ROS production (e.g., cytokines and radiation), and antioxidants can effectively block NF-kB activation in response to such stimuli, ROS were once implicated as central mediators in the NF-kB activation process (Schmidt, 1996).

However, more recent studies have failed to provide support for this hypothesis, finding that (1) activation of NF-kB in response to hydrogen peroxide is cell type specific; (2) an increase in ROS is not required for NF-kB activation in many instances; and (3) antioxidants may inhibit NF-kB activity through mechanisms distinct from redox regulation. Nonetheless, in certain cell types, oxidative stress is a potent activator of NF-kB and this can have important consequences for cell survival.

How do ROS activate NF-kB? The predominant mechanism by which NF-kB is activated by various stimuli is through the phosphorilation of IkB. IkB is an inhibitory protein that under normal conditions binds to NF-kB and sequesters it in the cytoplasm, thereby preventing its access to DNA. The phosphorilation of IkB results in its ubiquitination and degradation, freeing NF-kB to translocate to the nucleus and activate transcription through sequence-specific binding to DNA. For most inducers of NF-kB activation, IkB phosphorilation occurs on serine residues S32 and S36, and a number of different kinases have been reported to phosphorilate IkB on these sites. These include Ik-kinase (IKK), NF-kB-inducing kinase (NIK), double-stranded RNA-activated serine-threonine protein kinase (PKR) (Schoonbroodt and Piette, 2000), and Akt (Madrid, 2001). Many of these kinases offer obvious points for cross talk with other signaling pathways known to be activated by oxidant injury.

Virtually every step of the NF-kB signaling cascade is comprised of redox-sensitive proteins whose activities are modulated upon changes in ROS, some of these in a negative fashion (Schoonbroodt and Piette, 2000).

A large number of NF-kB dependent genes have antiapoptotic functions. These include TNF receptor-associated factor 1 (TRAF1), TRAF2, cellular inhibitors of apoptosis proteins (CIAPs) ,MnSOD, and A20 zinc finger protein (Bours , 2000).

NF-kB is also involved in regulating the expression of Bf1-1/A1 and Bcl-XL, two anti-apoptotic members of the Bcl2 family. Accordingly, NF-kB expression has been shown to exert protective effects under various conditions. However, NF-kB activity has also been correlated with apoptosis and the activation of apoptosis-associated genes such as Fas ligand and p53 (Kasibhatla, 1999). Studies examining the influence of NF-kB on cell survival following oxidant injury have likewise produced mixed findings. Although some studies have provided evidence for a protective function of NF-kB in response to oxidative stress (Kim, 2001) more studies support

the notion that NF-kB exerts a pro-apoptotic effect following oxidant injury (Aoki, 2001). In addition, the finding that p53-mediated cell death depends on NF-kB supports such a pro-apoptotic function (Ryan, 2000). Finally, it is worth noting that there are cases in which NF-kB has been shown to be activated in response to oxidant injury, but with no apparent influence on survival (Wang, 1998).

2. THESIS RATIONALE

2.1. *In silico* structure of MGC14832

The hypothetical product of c17-ORF37 gene, MGC14832 protein (also known as ORB3, XTP4 or C35) doesn't show similarity with other known proteins, but it has anyway kept our attention for the characteristics emerging from the *in silico* structure predicted with Gene Scan/Gene Wyse analysis.

Its sequences shows a total 89% homology with murine analogue as it is expressed in a region completely conserved (Katoh & Katoh, 2003); it is a 115 aa protein with a molecular weight of 12Kda, with a not-globular structure, high idrophylicy (18 Gln), and a putative complete intracellular structure. Besides, it presents many phosphorilation sites, PK cGMP, cAMP and Casein Kinase II dependent. Another relevant aspect is that it presents a putative CAAX box (CVIL) at its C-terminal, that is a farnesylation/geranylgeranylation site (prenylation site). This prenylation site is characteristic of many proteins involved in cell division (centromer associated proteins: CENP-E, CENP-F), and most of all it is essential for proto-oncogene Ras activation, witch deserves of being located in the internal surface of cytoplasmatic membrane to assert its mitogenic potential.

Making a comparative analysis of MCG14832 protein structure (http://www.ncbi.nlm.nih.gov/BLAST) we saw that it is strictly associated with Selenium W-related protein: this is a family found in both bacteria and animals, including the animal proteins SelT, SelW, and SelH, all of which are selenoproteins. These proteins contain a domain with a CXXC motif near the N-terminus, where selenocysteine may replace the second Cys, and levels of this selenoprotein are affected by selenium. The precise role of this family is unclear but it seems to be a protein family related with cell redox homeostasis.

3. AIM

We don’t know yet if c17-ORF37 gene product is simply a by-product of HER2 amplification or if this protein really exists and may contribute to clinical behaviour of HER2 positive tumours. We’ll try to know if the transcript from MGC14832 gene is only a by-product of HER2 amplification or if it is someway involved in tumour progression and resistance to therapy.

Furthermore we’ll try to find a correlation with c17-ORF37 transcript and some cellular pathways to give c17-ORF37 a function and involvement in tumour progression, founding on the *in silico* structure of the protein.

To understand this, it is necessary to acquire basic informations about transcript and protein expression on cancer cell lines, witch genetic aberration are highly representative of that occurring in primary tumours.

4. MATERIAL AND METHODS

4.1. Cell lines

SKBr3, BT 474: epithelial cells isolated and established from human breast cancer with HER2 amplification/overexpression.

ZR75-1, MB MDA 231: epithelial cell isolated and established from human breast cancer without HER2 amplification/overexpression.

All these cells are a kind gift of Dott. Michele Milella (Istituto Regina Elena, Roma).

293: human embryo kidney cell line, especially suggested for transformation study (kind gift of Dr. A. Houghton, Memorial Sloan Kettering Cancer Center).

These cell lines were all maintained in Dulbecco's modified Eagles medium (D-MEM) (Cambrex) supplemented with 10% foetal bovine serum (FBS) (Cambrex) and 0.5% penicillin/streptomycin.

NIH3T3: murine fibroblast cell line purchased from ATCC (American Type Culture Collection) were maintained in D-MEM supplemented with 10% calf serum (CS) (Cambrex) and 0.5% penicillin/streptomycin.

T9-4: murine fibroblast cell line derived from NIH3T3 with established transfection for the human oncogene HER2.

T19-7: murine fibroblast cell line derived from NIH3T3 with established transfection for the chimeric protein bearing the extracellular domain of human EGFR and the intracellular domain of human HER2.

They were maintained in D-MEM supplemented with 10% FBS (Cambrex) and 0.5% penicillin/streptomycin, and are a kind gift of Dott. Oreste Segatto (Istituto Regina Elena)

All these cell lines were maintained at 37°C in a humidified 5% CO_2 environment .

4.2. RNA extraction

Total RNA was isolated from cell lines with Nucleospin RNA II (Macherey Nagel) following manufacturer instruction witch included a treatment with RNase Free DNase I directly in column.

RNA yield was determined spectrophotometrically by measuring absorbance at 260nm and RNA integrity was confirmed by electrophoresis on a 1.2% agarose gel.

4.3. Quantitative Real Time PCR

Total RNA from cell lines, were used to measure total c17-ORF37 transcript. RNA were reverse transcribed using reagents supplied in RevertAid® H Minus First Strand cDNA Synthesis kit. 1μg of total RNA was denatured for 5 minutes at 70°C, followed by chilling on ice for 1 minute. The denatured RNA was incubated for 1 hour at 42°C in 20μl of a reaction mixture containing: 2μl of 10X RT buffer, 0.5 μg/μl of Oligo dT primers, 10mM dNTPs mix, 40 units Rnase inhibitor and 200 units of reverse trascriptase. Reactions were heated to 70°C for 10 minutes to inactivate the enzyme and then chilled on ice.

The PCR reaction was monitored in a MX3000P Personal Quantitative PCR System (Stratagene) using a Syber Green Fluorescent Master Mix (Stratagene). We used two couples of primers for c17-ORF37 detection on mouse samples and other two for detection on human ones: the first couple of primers was specific for mouse or human c17-ORF37 mRNA sequence, respectively, and the other couple was specific for the housekeeping gene β-actin (mouse) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), (human), used to normalize the expression level of c17-ORF37 gene.

Primers sequences are listed below:
Mouse:
mORF37 Fw: ATGAGTGGGGAGCCAGCG
mORF37 Rev: TCACAGGATGACACAGGGAGG
β-actin Fw: TTCGTTGCCGGTCCACA
β-actin Rev: ACCAGCGCAGCGATATCG
Human:
hORF37 Fw: ACAGGTGCCTTTGAGATAGAGATAA
hORF37 Rev: CTGTTGGTGATCTTTTCTAGGGTTT

GAPDH Fw: CAACAGCGACACCCACTCCT
GAPDH Rev: AGGCCATGTGGGCCATGA

Two different PCR cycles were used for mouse and human samples respectively, optimized to have 85-115% efficiency for each sets of primers.
PCR programs consisted of a hot start at 95°C for 10 minutes, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 62°C (for mouse samples) or at 63°C (for human samples) for 1 minute, and elongation at 72°C for 1 min. The program ends with a melting curve witch consist of a rapid renaturation at 45°C and a slow denaturation until 95°C in witch the fluorescence is read continuously to detect the melting point of eventually different double stranded DNA amplified. With this method we were able to verify that both the reaction amplified only a single fragment of DNA. All PCR amplifications were performed in triplicate samples.
Quantitative analysis of data was performed using the Mx Software and values from positive or treated cell lines or tissue were calibrated on the expression level of negative or control samples. Results shown are an average of at least three different experiments.

4.4. Preparation of a mouse c17-ORF37 transcript probe for Northern Blotting

1μg of total RNA from a mouse cell line (T9-4) was reverse transcribed using the RevertAid® H Minus First Strand cDNA Synthesis kit. The entire 350 bp sequence of mouse c17-ORF 37 was amplified by PCR using the following primers:

Sense: ATGAGTGGGGAGCCAGCG
Antisense: TCACAGGATGACACAGGGAGG

PCR was performed with a denaturation at 95°C for 10 min followed by 35 cycles at 95ºC for 1 min, 62ºC for 1 min, 72ºC for 1 min, with a final cycle at 72ºC for 10 min.

PCR products were purified using Nucleospin Extract II (Macherey Nagel) and cloned into pGEM-T Easy vector (Promega) using standard techniques to create pGEM-mORF37.

Murine c17-ORF37 sense and antisense digoxigenin-labeled RNA probes were generated from pGEM-mORF37. Briefly, the plasmid was linearized with either NcoI (antisense) or SalI (sense) and purified with the Nucleospin Extract II (Macherey Nagel). Plasmid (1μg) was incubated for 5 hours at 37°C with 10X transcription buffer (Roche Applied Science), digoxigenin-labeled nucleotide mix, 40 units/μl RNAsi inhibitor, and SP6 or T7 RNA polymerase (Roche Applied Science), respectively. The RNA probes were precipitated overnight at -20°C in 60μl 100% EtOH, 2μl 4M LiCl, 2μl 200mM EDTA, centrifuged at 12.000 x g for 15 minutes at 4°C and resuspended in DEPC H_2O. Colorimetric analysis was then performed to estimate the probe yield.

4.5. Northern Blotting analysis

Total RNA (10μg), purified as described above, were separated on a denaturing 1.2% agarose gel containing 1X MOPS and 6.7% formaldehyde. The gel was washed for 10 minutes in 20X SSC to remove the formaldehyde before transfer to a Nytran Plus membrane (Schleicher and Schuell).

To immobilize the RNA, the nylon membrane was subsequently exposed to UV ray for 1min 30 sec. Pre-hybridisation of the membrane was performed in DIG-Easy-Hyb buffer (Roche Applied Science) at 63°C for 1 hour. For the hybridisation, 10 ml of DIG-Easy-Hyb, to which had been added the DIG-labeled RNA probes (100 ng/ml) or β-actin probe (30 ng/ml; Roche Applied Science), were used per 100 cm^2 of membrane. The hybridization was occurred at 63°C for 16 hours.

The membrane was washed twice with 2X SSC / 0.1% SDS for 5 minutes and twice with 0.1X SSC / 0.1% SDS for 15 minutes at 63°C. The membrane was washed with Buffer 1 (100 mM Maleic acid, 150 mM NaCl, pH 7.5) for 5 minutes at room temperature.

To detect the RNA-RNA hybrid, the membrane was preincubated for 45 minutes in blocking solution (Buffer 1 + 1% (w/v) casein) at room temperature and then incubated for 30 minutes with anti-DIG-antibody AP conjugate (Roche Applied

Science) diluted 1:10.000. A 2 x 15 minutes washing step in Buffer 1 was followed by equilibration in detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl2) for 5 minutes. Detection was performed with CSPD (Roche Applied Science). Finally, the membrane was exposed on Lumi-film chemiluminescent detection film (Roche Applied Science) for 2 hours or overnight.

4.6. Cell lysates

Cultured cells were collected by low-speed centrifugation at room temperature for 5 minutes. After removal of culture media, the cell pellet was washed twice with cold PBS. Whole lysates were prepared by incubating 10^7 cells per ml Ripa buffer (25mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Na deoxicolate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl flouride PMSF, 1mM Na-Vanadate, 2μg/ml aprotinin, 1μg/ml leupeptin and 1μg/ml pepstatin) for 30 minutes on ice. Lysates were cleared by centrifugation at 12.000 x g for 10 minutes at 4°C. The supernatant fluid was transferred to a new microfuge tube and the pellet was discarded. Protein concentration was determined by Bradford protein assay (Biorad).

4.7. recORF37 recombinant protein: Gateway Cloning Technology

Gateway Cloning Technology (Invitrogen) is a powerful methodology that facilitates protein expression. Using this technique it is possible to transfer DNA segments between vectors. In the first reaction (the BP reaction) a PCR product flanked by AttB recombinant sites is cloned into a Donor Vector to produce an “Entry Clone” in witch the gene of interest became flanked by an AttL site. In the second step, the LR reaction, the Entry Clone undergoes recombination via the AttL sites with a Destination Vector to create an Expression Clone.
Murine c17-ORF37 DNA sequence was amplified from pGEM-mORF37. PCR program was performed as described above.
Primers used were:

attB1Fw:GGGGACAAGTTTGTACAAAAAAGC AGGCTTGAGTGGGGAGCCAGCG

attB2Rev:GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACAGGATGACACAGG GAGG

Using these primers we obtained a PCR product flanked by attB recombination sites to generate the mORF37 Entry Clone.
The PCR product was purified using Nucleospin Extract II (Macherey Nagel).The BP Reaction was set up using pDonor201 Vector, BP Clonase Enzyme Mix, BP Reaction Buffer and AttB-PCR product,following manufacturer's instruction, and was incubated for 3 hours at 25º. Proteinase K solution (37°C for 10 min) was added to stop the reaction.
2 μl of BP Reaction was used to transform DH5α electro-competent cells and they were spread on LB agar + 50μg/ml Kanamycin plates.
Entry Clone plasmids from spread colonies were extracted using GenElute Plasmid Mini Kit (Sigma) and screened for the presence of the insert by PCR using attL primers.

AttL1:TCGCGTTAACGCTAGCATGGATCTC
AttL2:GTAACATCAGAGATTTTGAGACAC

The correct Entry clone was confirmed by sequencing using the mentioned attL primers, using manufacturer's PCR program.
An LR Reaction, was performed between the Entry Clone and a the Destination Vector pET-DEST17: LR Reaction Buffer, Entry Clone, Destination Vector and LR Clonase Enzyme Mix were incubated at 25°C for 3 hours. Proteinase K was added to stop the reaction. 1μl of the LR Reaction was used to transform BL21 Star (DE3) competent cells.
This strain carries a mutated *rne* gene (*rne131*) which encodes a truncated RNase E enzyme that lacks the ability to degrade mRNA and this results in increased mRNA stability. These cells are also deficient in the outer membrane protease, that is important to reduce degradation of proteins expressed in the strains.
Transformed BL21 Star were spread on LB agar + 50μg/ml Ampicillin.

The plasmid was extracted from colonies and checked by PCR screening using the mentioned above attB primers.

4.8. Expression and purification of recORF37 recombinant protein

The BL21 Star (DE3) E. Coli strains are chemically competent cells suitable for high level recombinant protein expression.

Positive transformants were cultured in LB medium containing Ampicillin and growth at 37°C over night (mid log phase) and then diluted to allow the cells to return to logarithmic growth. The cells were induced for 3 hours with 0.3M NaCl since they reached mid-log phase (OD_{600}= 0.5-1). Effectiveness of induction was confirmed by SDS PAGE and Comassie staining, with an equal amount of not inducted (NI) and inducted (I) cell lysates run on gel. A new protein band with the expected MW=12.000 appears on the "I" culture.

Cell pellets from BL21 transformants were suspended in binding buffer (sodium phosphate buffer 20mM, NaCl 0.5M) pH=7.8, lysozyme (1mg/ml) was added and the suspension was incubated on ice for 30 min. After a 10 min incubation at 4°C, TritonX100 (1X final), DNase (5μg/ml) and RNase (5μg/ml) were added and lysates incubated for further 15 min. on ice.

The suspension was centrifuged at 20.000 rpm for 20 min and the supernatant was filtered (0.45μm) before loading on the column.

HiTrap Chelating HP, 1ml column (Amersham Biosciences) was used to purify recORF37 recombinant protein. Prior to use, the column was washed with 5 ml distilled water. 1ml of metal salt solution (0.1M $NiSO_4$) in distilled water was loaded on the column. After these steps the column was washed again with distilled water.

After column preparation, it was equilibrated by washing with 5-10 column volumes of binding buffer (0.02M sodium phosphate, 0.5M NaCl pH=7.8). The sample was applied using a pump and the flow rate was 1ml/min. After loading and washing with 5-10 column volumes of binding buffer, recORF37 protein was eluted using a pH steps. 5 ml of phosphate buffer of each pH (ranging 7.8 to 4.0) was loaded on the column. A FPLC (Mono Q) column was used to further purify recORF37 protein using a phosphate buffer (0.02M, pH=7) and a NaCl gradient.

4.9. Mice and Rabbits immunization for anti-MGC14832 antibody production

Mice or rabbits were immunized with pcDNA3.1 mORF37 (see chapter 4.15. for plasmid contruction) to obtain specific antibodies: in brief animals received a priming and two boosts with 100μg of plasmid in 100μl of saline, with intramuscular injection every 15 days. A week post the last boost blood was collected and sera were assayed for antibody production.

In collaboration with Genesis Biotech Inc. (Taiwan) we immunized rabbits to obtain anti-human or mouse c17-ORF37 antibody.

Anti-human c17-ORF37 antibody were obtained immunizing animals, with a priming and two boosts subcutaneously, with a synthetic peptide encompassing amminoacid 4-22 from human sequence (4-EPGQTSVAPPPEEVEPGSG-22). Peptide was successfully synthesized, checked at mass spectrometry, and fused to KLH. Keyhole limpet hemocyanin (KLH) is a respiratory protein found in mollusks. Its large size makes it very immunogenic, and the large number of lysine residues available for conjugation make it very useful as a carrier for haptens. The phylogenic separation between mammals and mollusks increases the immunogenicity and reduces the risk of cross-reactivity between antibodies against the KLH carrier and naturally occurring proteins in mammalian samples.

Succinylated KLH was conjugated to the hapten, via cross-linking with carbodiimide between the newly introduced carboxyl groups of KLH and the amine groups of the hapten. Rabbits were immunized with a priming (using complete Freund's adjuvant, 1:1 ratio) a two boosts (using incomplete Freund's adjuivant, 1:1 ratio), subcutaneously at multiple sites, with 300 μg of the fused peptide.

Anti-mouse c17-ORF37 antibody were obtained immunizing animals with a priming intrasplenic injection of 100 μg of pcDNA3.1 mORF37 as described (Velikovsky, 2000), and boosting with subcutaneous injection of the recombinant protein recORF37 (300 μg, 1:1 ratio with complete and incomplete Freund's adjuvant).

Sera were collected from the immunized animals and affinity purified with a Protein A sepharose column (Amersham). The same procedure was followed with the pre-immunization sera.

4.10. Hens immunization and IgY isolation from egg yolk

To produce antibodies raised against the putative protein MGC14832, laying hens, obtained from a local egg farm, were injected subcutaneously at multiple sites with 300 μg of recORF37 using complete Freund's adjuvant at first injection an incomplete Freund's adjuvant for following injection (1:1 ratio), at days 1, 14 and 21. When a suitable number of eggs had been collected IgY antibodies were extracted from the yolk according to Concetti, 1994. Briefly, yolks were separated from the whites and broken in 4 volumes of 10mM phosphate buffer pH=7.5, containing 0.1 M NaCl. After stirring PEG 6000 powder (Sigma) was slowly added at a final concentration of 3.5%. The precipitate obtained was discarded after centrifugation at 17.000 rpm for 10 min and supernatant made 12% PEG. The proteins, pelleted at 17.000 rpm and redissolved in 15mM phosphate buffer pH=8, were further purified using a DEAE chromatography column and eluted in the same buffer plus 0.3M NaCl. The same procedure was followed for some egg collected before injection, from witch we obtained pre-immune IgY. Protein concentration was determined by Bradford protein assay (Biorad).

4.11. Western Blotting analysis

Electrophoretic assays of cell lysates or recombinant protein were performed using the Bio-Rad electrophoretic system. Protein samples in loading buffer were heated at 100°C for 5 minutes and loaded on 15% SDS-PAGE. After electrophoresis and electro-transfer, the nitrocellulose membrane (PROTRAN Schleicher & Schuell) was blocked with 1% non fat-dry milk (Bio-Rad) in TBS-T for 1 hour. The membranes were probed with selected antibodies or animal sera for 1 hour at 37°C or overnight at 4°C. Bound antibodies were detected using appropriate secondary HRP-conjugated antibodies (Calbiochem) for 1 hour at 37°C or overnight at 4°C, and visualized with a commercial ECL kit (Amersham).

4.12. ELISA

ELISAs were performed according to standard protocols. In brief, recombinant protein (100 ng/well) was coated in a 96 wells plates in carbonate buffer pH 9.6 for 1 hour at 37°C. After incubation with primary antibodies or antisera (indicated dilution) for 1 hour at 37°C and reaction with HRP-conjugated secondary antibody (1:3000) for 1 hour at 37°C, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Sigma), was added as substrate, and the developed color read at 405 nm.

4.13. EMSA (electrophoretic mobility shift assay)

Nuclear extracts are prepared as described (Zhou, 2000) with some modifications: in brief cells were lysed gently in a NP-40 containing sucrose buffer, while the nuclei remain intact. After a washing step they were resuspended in an ipotonic low salt buffer: the nuclei swell. Then a high salt buffer was added slowly: the nucleoplasm was extracted into the buffer. The extract was separated from nuclear envelope and DNA by centrifugation.

To asses NF-kB activation 5μg of nuclear extract were run on a 6% non denaturing PA (O.5x TBE) gel, at 100V, together with NF-kB binding site double stranded biotin-labeled or cold DNA (customized from Operon):

NF-kB sense: AGTTGAGGGGACTTTCCCAGGC
NF-kB antisense:GCCTGGGAAAGTCCCCTCAACT

Run transfer and detection followed manufactures instruction of LightShift Chemiluminescent EMSA Kit (Pierce). Reactions to be performed include a negative control with no protein extraction for DNA to bind: no shift is observed and it establishes the position of unshifted probe band. The experimental samples, and the experimental samples with 200-fold unlabeled DNA were run to demonstrate that the shifted signal can be prevented by competition, and that the shift results from specific protein-DNA interaction.

A supershift band is obtained running samples with anti p50 or p65 NF-kB antibody

(Santa Cruz): specific antibody bound to NF-kB-DNA complex causes a further delay in DNA run.

4.14. Cell treatment with PI3K inhibitors

Cells were plated at $5 \cdot 10^5$ cells/well in complete medium in a 6 wells plate and let to adhere over-night. The following day medium was changed in medium without serum (starvation medium) and PI3K inhibitors were added at the following concentrations: Wortmannin (Sigma), 100nM, Gö6976 (Calbiochem), 10μM, Ly294-002 (Calbiochem), 100nM. Cells were incubated at 37°C in a CO_2 incubator for 24h and then harvested for RNA extraction, cDNA transcription and quantitative Real Time c17-ORF37 transcript quantitation.

4.15. Stable transfection of NIH3T3 with murine c17-ORF37

pcDNA3.1 (Invitrogen) has been chosen as the expression vector for stable transfection of NIH3T3 with the entire sequence of mouse c17-ORF37, as it carries the *neo* gene, responsible of antibiotic resistance to the selective antibiotic geneticin. pcDNA3.1 without the insert was included as negative control of transfected cells.
For the generation of the plasmid mORF37 was first amplified with a couple of primers flanked by EcoRI and HindIII digestion sites, using pGEM-mORF37 as a template.

HindIII Fw: GCATAAGCTTACCATGAGTGGGGAGCCAGCG
EcoRI Rev: GGTGGAATTCTCACAGGATGACACAGGGAGG

The amplified product was digested with HindIII and EcoRI and cloned into the HindIII-EcoRI cloning sites of pcDNA3.1 vector.
Plasmid was linearized with the restriction enzyme BglII, to favourite integration, and purified with Nucleospin Extract II (Macherey Nagel) kit; concentration of DNA was determined spectrophotometrically by measuring absorbance at 260 nm, and if necessary brought to the final concentration of 1μg/μl.

NIH 3T3 cells were cultured in normal growing medium. The day before transfection, they were trypsinized, counted and plated in 24 wells plates at 1.5 X 10^5 cells per well so that they are 90-95% confluent on the day of transfection. Cells were plated in 0.5 ml of their normal growth medium containing serum and without antibiotics.
For each well of cells to be transfected (one with pcDNA 3.1-mORF37 and another with the empty vector pcDNA3.1) 0.8 µg of DNA was diluted into 50µl of OPTI-MEM I Reduced Serum Medium (Invitrogen) without serum, and 2µl of LIPOFECTAMINE 2000 (LF2000™) Reagent (Invitrogen) was diluted into 50µl OPTI-MEM I Medium and incubated for 5 min at room temperature. DNA and LF2000 were then mixed and incubated at room temperature for 20min to allow complexes to form.
The DNA-LF2000 Reagent complexes (100µl) was added directly to each well, from witch growth medium had been removed and changed in medium without serum, and incubated at 37°C in a CO_2 incubator for 4-5 h. Growth medium has been replaced after 4-5 h with complete medium. 24-48 hours post transfection cells were diluted 1:20 in two 96 wells plates and selective antibiotic (Geneticin, Invitrogen) was added at a final concentration of 500µg/ml. Medium plus selector was changed every 3 days until it was possible to isolate single stable transfected clones.
The clones isolated were tested by quantitative Real Time PCR for murine c17-ORF37 expression using as calibrator NIH3T3 transfected with empty vector.

4.16. Transient transfection of 293 with GFP-mORF37

pVAX1 (Invitrogen) has been chosen as the expression vector for transient transfection of 293 with the entire sequence of mouse c17-ORF37 fused at its N-terminus with green fluorescence protein (GFP) from the jellyfish *Aecquorea Victoria*.
For the generation of the intermediate plasmid, GFP was first amplified with a couple of primers flanked by HindIII and BamHI digestion sites, using pGFP (Clontech), as a template.

HindIII-GFP-Fw: GCATAAGCTTACCATGAGTAAAGGAGAAGAACTT
BamHI-GFP- Rev: GGTGGGATCCTTTGTATAGTTCATCCAATGCC

The amplified product was digested with HindIII and BamHI and cloned into the HindIII-BamHI cloning sites of pVAX1 vector. The plasmid obtained was verified by sequencing. Murine c17-ORF37 was amplified with an oligo flanked by BamHI digestion site and EcoRI Rev (described above).

BamHI Fw :GCATGGATCCAGTGGGGAGCCAGCGCCGGTG

The amplified product was digested with BamHI and EcoRI and cloned in pVAX-GFP intermediate plasmid, to obtain the fusion product pVAX-GFP-mORF37. The plasmid was checked by sequencing.
For transient transfection of 293, it was used the same protocol as for stable transfection of NIH3T3, scaling up volumes and quantity for a 6-wells plate following manufacturer instruction. Transfection was performed on cells grown on poly-lysined cover slip, on 6 wells plate, and 24h post transfection, they were analysed for fusion protein expression and subcellular localization on confocal laser microscope (Biorad MRC600).As a control for specific fusion protein localization, 293 cell line was transfected with pGFP plasmid, following the same protocol.

4.17. MTT assay for cell proliferation/viability

Cell were plated in 96 wells plates at the indicated number in quadruplicate and let to adhere over-night. The following day they were treated with appropriate chemicals and incubated at 37°C in a CO_2 incubator for the indicated period of time. At the end of treatment 20μl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were added per well (final concentration: 0.5 mg/ml) and plates were incubated for further 2-3 h. Colour was developed after flicking and tapping the plate and cell disruption with 100 μl dimethyl sulfoxide (DMSO, Sigma) and read on a multiwell scanning spectrophotometer (ELISA reader) at 540 nm. Data reported are an average of at least 3 different experiments.

4.18. Cytotoxicity Assay for cell necrosis

With MTT assay we are only able to measure viable cells, but we are not able to distinguish between apoptotic or necrotic ones. At this purpose we measured lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis, in supernatant medium of treated cells; assay was performed by Citotox 96-Non radioactive Cytotoxicity Assay (Promega). The test consists in a coupled enzymatic assay witch results in the conversion of a tetrazolium salt (INT) into a red formazan product. In brief cells were treated as described above; at the end of treatment 96 wells plate was centrifuged at 1600 rpm for 5 min., and 50 μl of supernatant were transferred to a new 96 wells enzymatic (ELISA) plate. 50 μl of Substrate solution were added and plate was incubated 30 min. in the dark. 50 μl of stop solution were subsequently added and colour formed was read at 490 nm on a multiwell scanning spectrophotometer (ELISA reader). Cells from the previously centrifuged plate were tested for they overall viability with described MTT assay.
Necrotic cells (experimental value) were counted as a percentage on spontaneous LDH release (not treated cells) and maximum release, obtained lysing the cell with a 0,8% (final concentration) solution of Triton X-100 for 45 min.

$$\frac{Exp - Spont}{Max} \cdot 100 = \%Necrosis$$

4.19. Peroxidase activity assays

In order to asses if recORF37 purified recombinant protein, and/or mORF37 transfected cells possesses a peroxidase activity we performed a series of peroxidase activity assays. Transfected and control NIH 3T3 were lysed by multiple freeze and thawing in H_2O in order to maintain the proteins intact at a concentration of 10^7 cells/ ml .

Catalase like assay:

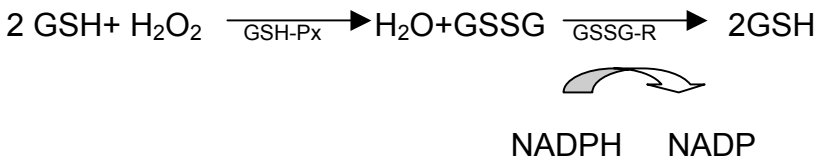
This method is based simply on the measurement of the loss of absorbance at 240 nm of eventually metabolised H_2O_2.

Chemiluminescent assay:

Chemiluminescent measurements were performed on Automat Berthold LB 953 (Berthold Co. ,Wilbard, Germany). Assay was performed at different concentrations of purified recombinant protein or cell lysates in a final volume of 1 ml of PBS containing 100 μM luminol. Reaction started after injection of 50 μl of H_2O_2 (22mM final concentration). Measurement was followed for 40 sec. and area was calculated as photon per second (cps).

Glutathione dependent peroxidase assay:

This method measures glutathione (GSH) oxidation degree by H_2O_2, catalysed from glutathione peroxidase (GSH-Px). This substrate is maintained constant adding glutathione reductase (GSSG-R) and NADPH, witch immediately convert each molecule of oxidised glutathione (GSSG) in its reduced form oxidising NADPH to NADP:



Reduced glutathione formation (GSSG) is measured following the loss of absorbance, at 340 nm, of NADP.

4.20. Höechst 33258 nuclear staining

After H_2O_2 treatment (300 μM for 2 h) transfected NIH3T3 were washed two times with PBS, and fixed with 4% paraformaldehyde for 10 min. at room temperature, washed with PBS and incubated with 10 μg/ml Hoechst 33258 (Sigma), for 10 min in the dark. Nuclear morphology was observed under fluorescent microscope (Olympus AX80).

4.21. JC-1 staining for mitochondrial potential

NIH3T3 transfected for mORF37 or not, were treated with 300 μM H_2O_2 for 2 h in

starvation medium. After treatment serum was added to the cells to reach 1% final concentration. JC-1 (Invitrogen) was added at a final concentration of 10 μg/ml, mixed thoroughly to avoid coloured aggregate formation, and incubated for 30 min. at 37°C. Cells have been washed two times in PBS and observed under fluorescent microscope (Olympus AX80), to detect mitochondrial depolarisation (increase in green/red fluorescence intensity ratio) that indicates early stages of apoptosis.

4.22. Apoptosis detection by Annexin V

Transfected NIH3T3 were treated with 50 μM H_2O_2 for 6h in medium without serum, then were trypsinized, and washed two times with PBS. $3 \cdot 10^5$ cells from treated or untreated control cells were resuspended in 195 μl binding buffer (10mM HEPES/NaOH, pH=7,4, 140 mM NaCl, 2,5 mM $CaCl_2$). 5 μl Annexin V (Alexis) were added to each sample for 10 min. at room temperature in the dark. Cells were then washed and analysed by flow cytometry (Becton Dickinson).

5. RESULTS

5.1. c17-ORF 37 transcript correlates with HER2 amplification

To evaluate a possible correlation between c17-ORF37 transcript and either HER2 gene amplification or HER2 protein expression, we performed a quantitative Real Time PCR using a c-DNA from human breast cancer cell lines with different HER2 status. Both BT474 and SKBr3 present HER2 amplification with the former having a higher estimated HER2 gene copy number than SKBr3 (16 copy vs. 32, Tomasetto, 1995) but with HER2 protein inversely overexpressed (Tomasetto, 1995 and fig.1). MB MDA 231 and ZR 75-1 were chosen as breast control tumour cells with no HER2 gene amplification and with basal protein expression (fig.1).

Real Time PCR results reported in fig.2, show that the level of c17-ORF37 transcript correlates more tightly with HER2 amplification than with protein overexpression. The same association was found when cells had been kept in starvation (serum free medium) (fig.3).

These results are in accordance with previous work, (Kauraniemi, 2003) that shows, by Real Time PCR analysis, an increase in the number of c17-ORF 37 transcript on frozen tumour tissues with HER2 amplification.

5.2. Antibody production against MGC14832

In order to detect the c17-ORF37 gene product, MGC14832 protein, we needed a suitable tool such as specific anti-MGC14832 antibodies, that we tried to produce, in our laboratory and in collaboration with Genesis Biotech Inc. (Taiwan). In spite of the very different approaches used, we had difficulties in breaking the immunotolerance in our models, probably because this hypothetical protein is largely conserved among various animal species (Katoh & Katoh, 2003).

As described in Materials and Methods, we prepared a full-length mouse c17-ORF37, eukaryotic expression vector, for DNA immunization, which failed to give an antibody response both in mice and rabbits (data not shown).

In second instance, by using Gateway Cloning Technology (Invitrogen), we produced

and purified the entire mouse recombinant protein (named recORF37) (fig.4).

In collaboration with Genesis Biotech Inc. (Taiwan) we used recORF37 to immunize rabbits: in brief rabbits were first primed with the full-length mouse c17-ORF37 eukaryotic expression vector, by intrasplenic injection, thought to induce a quicker response producing a larger number of B activated clones, then boosted subcutaneously with the recombinant protein as described in Materials and Methods.

Antisera were collected and the affinity purified antibody (named GA427) was found to react against the recombinant protein both in Western Blotting and in ELISA at very high dilutions (fig.5-6), but was unable to reveal specific signals either on mouse or on human cell lysates, supposed to contain the expressed c17-ORF37 protein.

To overcome the obstacle of the close homology of MGC14832, between mouse, rat and human, we decided to use laying hens as host for eliciting anti-MGC14832 antibodies, since birds are known to give good immunoresponse to mammalian antigens.

Chickens were then immunized with the recombinant protein as described in Materials and Methods, and total IgYs obtained from egg yolk were tested for the reaction against the same protein. Chicken antibodies worked well both in Western Blot and in ELISA with the recombinant protein at very high dilutions (fig.7-8), but again no specific signals were detected on cell lysates.

In order to overcome immunotolerance and to enhance the specificity of the antibody to be produced, researchers from Genesis Biotech Inc. (Taiwan) synthesize the peptide encompassing the amminoacidic sequence 4-22 of human c17-ORF37, as this protein portion shares no similarity with any other protein in human proteome, (Table A).

Peptide 4-22 was successfully synthesized, its sequence verified by mass spectrometry, and this epitope fused with KLH (Keyhole limpet hemocyanin) by standards method, and finally injected in rabbits following the schedule reported in Materials and Methods. The affinity purified polyclonal antibody obtained (named GA486) was able to detect the human cognate peptide (fig.9).

When this antibody was used in Western Blotting analysis against human breast cancer cells lysates (fig.10) we obtained a strong signal at the expected molecular weight of 12 KDa, not detected with preimmune serum. This antibody failed to react

with mouse cell lysates supposed to express murine c17-ORF37 (see further).
We report for the first time on in vivo expression of MGC14832 protein, and show that the levels of the protein in the human tumour cell lines examined, parallel the mRNA quantities previously measured (fig.11).

5.3. c17-ORF 37 transcript and HER2 overexpression in mouse model

To better understand whether the relationship between HER2 protein overexpression and c17-ORF37 transcript levels was depending on gene amplification, we used mouse cells, as in mice HER2 and c17-ORF37 are located in a strictly conserved synthenic locus (Katoh & Katoh 2003), but are never amplified.
To this purpose, we used T9-4, a NIH3T3 mouse fibroblast cell line, transfected with the human HER2 gene, which is then overexpressed in absence of gene amplification.
By performing Northern Blotting analysis on these cells, we surprisingly found that c17-ORF37 mRNA is overexpressed in T9-4 cell line compared to parental cells, especially when they were maintained in starving medium, to indicate that the process is independent from external signals (growth factors, hormones etc) (fig.12).
To confirm and quantify our observation we performed a quantitative Real Time PCR, using, as a control of the transfection, T19-7, a NIH3T3 cell line transfected with the chimeric gene coding for the extracellular domain of EGFR (HER1) and the intracellular domain of HER2: the resulting chimeric protein is unable to be activated in the absence of EGF, its proper ligand.
We found the differential pattern of expression on c17-ORF37 transcript between T9-4 and NIH 3T3, already observed in Northern Blotting, when cells were grown in starving medium. When cells were grown in complete medium total level of c17-ORF37 transcript were comparable among the cell lines (fig.13). Furthermore, T19-7 have the same basal level of c17-ORF37 transcript as NIH 3T3, excluding a role of the transfection per se, in the observed overexpression.
In absence of external signals, NIH3T3 are no more able to transcribe for c17-ORF37, while T9-4 maintain the same level of transcript, also in the absence of

serum.
From these preliminary data it may be supposed that HER2 overexpression at protein level is sufficient for c17-ORF37 transactivation, independently on HER2 amplification status, at least in this model, and independently on transfection itself.
The lack of a suitable antibody did not allow the detection of murine c17-ORF37, at protein level.

5.4. c17-ORF 37 transcript can be modulated in a mouse model with some PI3K inhibitors

XTP4 is one of the c17-ORF37 protein alias (Gene Bank accession number: AF490253), reported to be the “human gene 4 transactivated by hepatitis B virus X antigen” (Liu, 2002, unpublished data, direct submission on NCBI).
As the inflammatory hepatitis B antigen, a potent viral oncogene, is capable to activate a large set of genes through NF-kB activation, we asked ourself whether c17-ORF37 could be activated likewise, by HER2 induced NF-kB activation.
As it is reported (Zhou, 2000), HER2 activates Akt and NF-kB without extracellular stimulation, and we confirmed, by Electromobility Shift Assay (EMSA), that NF-kB is activated on T9-4 cell line, in a constitutive manner due to HER2 overexpression, while in NIH3T3 cell line it is not (fig.14). From the data in fig.13 it is evident that c17-ORF37, on T9-4 cell line, is constitutively expressed in a steady state fashion, as no changes from starving are observed. On the contrary, starving conditions strongly reduced c17-ORF37 expression on NIH3T3.
Our attention focused on NF-kB, a nuclear factor activated under a series of stress conditions; it stimulates proliferation and blocks programmed cell death (apoptosis), in different cell types, including breast cancer. Activated NF-kB was detected predominantly in ER (estrogen receptor) negative vs ER positive breast cancer, and mostly in the subset of HER2 positive tumours (Biswas, 2004). NF-kB can activate several antiapoptotic genes (among this Bcl-2, Bcl-xL and c-FLIP) and can transactivate the cell cycle regulatory protein cyclin D1, witch causes increased phosphorilation of retinoblastoma protein, through PI3K/Akt activation (Biswas, 2000), and Akt activation strongly correlates with HER2 expression (Zhou, 2000, and

fig.14).

For all the reasons explained above, we tried to find out whether PI3K/NF-kB cascade could be involved in c17-ORF37 transactivation, using some of this pathway's inhibitors.

To this attempt we used some commercially available compounds: Wortmannin, an irreversible inhibitor of PI3K that doesn't affect upstream signal events (PI4K is inhibited at a concentration 100-fold higher), Gö6976 an inhibitor of Ca^{++} dependent PKC, and Ly 294-002, another PI3K inhibitor that act on the ATP binding site of the enzyme, not affecting EGFR, PKC, or PI4K activity (fig.15).

First of all, we treated T9-4 for 24h in starved medium, at the indicated concentration of inhibitors, and performed a quantitative Real Time PCR to evaluate changes in c17-ORF37 mRNA between treated and untreated cells. We found that both Wortmannin and Ly294-002 lead to a 60-70% decrease in the amount of c17-ORF37 transcript, while no differences between control cells and Gö6976 treated ones, were present (fig.16). Thus, we suggest that, at least in this model of HER2 transfected cells, c17-ORF37 is transactivated by means of PI3K pathway, without involvement of PKC activation.

On the basis of the above results, we went back to human tumour cells to investigate if and how PI3K pathway could be involved: none of the inhibitors, tested in mouse, affected the amount of c17-ORF37 transcript in human cells (fig.17).

What we can infer from this data is that the genetics of the tumour cell is much more complex than that of mouse transfection model. The real association of c17-ORF37 and HER2 protein expression, beyond gene amplification, remains to be evaluated.

Because of such a genetic complexity of human cancer cells, we chose to work with murine c17-ORF37 transfected fibroblast with the aim to have a much simpler model, on witch to study expression and function of MGC14832 protein.

5.5. NIH 3T3 transfection: "gain of function"

We transfected NIH3T3 cell line with mouse c17-ORF37 cDNA, and obtained a stable transfectant as verified by quantitative Real Time PCR for the overexpression of c17-ORF37 mRNA (fig.18). Among the positive clones, n°7 was selected as

expressing the highest level of c17-ORF37 transcript. The lack of a suitable antibody did not allow to detect the expression of murine c17-ORF37 (mORF37), at protein level.

To bypass this problem we transiently transfected 293 cell line with a vector encoding mouse c17-ORF37 protein fused at its N-terminus with the GFP (green fluorescent protein). 24h post transfection, cells, grown on polylysined cover slips, were fixed and checked at confocal laser microscopy, for protein expression and cellular localization.

Microscopy images (fig.19) confirmed the expression of c17-ORF37 in the transfectants, and its intracytoplasmatic location as hypothesized from *in silico* structure. The distribution of fluorescence inside the cell appears in a well characterised fashion, and seems not to be casually widespread in the cytoplasm. It appears in a defined internal distribution, with a “donuts like” morphology, mostly perimembrane. In some cases the fluorescence is very intense, condensed in some “vesicle like” structure. We don’t know if what we observe is due to the real distribution of the protein *in vivo*, or if it is due to the not natural overloaded system.

This last possibility was refused as fluorescence distribution in 293 cells transfected with pGFP plasmid, is casual and widespread in all the cell (fig.20).

5.6. MGC14832 as a survival factor?

As next step, utilizing the NIH3T3 mORF37 transfectant, we tried to find out some roles of the MGC14832 protein, moving from two major hypothesis: (I) one rising from our observation that the protein is transactivated via PI3K/NF-kB pathway, a pathway involved in cell survival and resistance to apoptosis to several extracellular injury, among which is the oxidative stress (Martindale, 2002); (II) the second one, rising from the *in silico* structure of MGC14832 which is supposed to be a Seleno-W related protein, belonging to a family of protein with peroxidase activity.

With this premises in mind we performed H_2O_2 toxicity assays, treating the cells with serial dilution of H_2O_2, from 640 μM to 10 μM, for 6 and 24 hours. Transfected mORF37 NIH 3T3 cells showed an increase in the overall viability, with a EC_{50} (effective concentration 50%) at 24 h of 250 μM, versus 80 μM of the empty vector transfected NIH 3T3 (fig.21-22, Table B).

We extended this study to an other oxidative agent such as tert-butyl-hydroperoxide, (t-BOOH) a peroxide radical generator: also with this compound mORF37 transfected cells earn an advantage on survival having an EC_{50}=250 μM versus 30 μM in the empty vector transfected cells (fig.23, Table B).

From these data a potential role of c17-ORF37 as a survival factor is arising.

To verify whether this advantage on cell survival against an exogenous oxidative stress was due to a peroxidase activity of the protein, we performed a series of enzymatic assays, both on the whole cell (transfected or not) not-denatured lysates, and on recORF37 recombinant protein.

The assays included the detection of catalase like activity, glutathione like peroxidase activity, peroxidase activity coupled to luminol oxidation (see Material and Methods).

None of the assays, summarized in Table B, gave positive results either on recombinant protein or on cell lysates.

As regarding to the recombinant protein, it could be argued that recORF37, produced in bacteria, lacks of secondary modifications that occur after protein synthesis and which may be essential to its enzymatic activity. Alternatively, the protein could have lost its proper folding during the long process of purification.

On the other hand, the large amount of other peroxidase (like catalase or glutathione peroxidase) might have hidden the possible activity due to mORF37, on cell lysates. So we can't rule out that MGC14832 is devoid of a peroxidase activity.

However, we wanted to understand in witch manner mORF37 transfected cells acquired an advantage on cell survival: did the control cells die for necrosis or apoptosis? Is MGC14832 able to modify cell susceptibly to oxidative stress avoiding apoptosis?

For the purpose, as an indicator of the loss of integrity of the cytoplasmatic membrane occurring during necrosis and not during apoptosis, we measured the LDH release from H_2O_2 treated cells (fig.24-25). The results show that necrosis is not present, at least at low concentrations or at the short time of treatment. At 6 h only a little percentage of cells undergoes necrosis. At 24 h, the percentage increases, but never reaches 100%, even at high concentration of H_2O_2 (320 or 640 μM).

When all the cells are no more viable, as revealed by MTT assay, only half of control or 20% of mORF37 transfected cells are LDH releasing. The data suggest a

protective role on the pathways of events leading to necrosis.

However, when the cells are treated with 80 μM H_2O_2 for 6 h, in which 60% of control cells, or 85% of mORF37 transfected ones are viable, necrosis is not observed, so it could be indirectly inferred a protective role of mORF37 also on the apoptotic pathway.

In order to directly ascertain such a role on apoptosis induced by H_2O_2 we performed the following experiments:

(I) nuclear staining with Höechst 33258 (fig.26-27), which reveals nuclear condensation and blebbing, as well as a decrease in nucleus/cytoplasm ratio in treated vs not treated control cells; these features are much less present in c17-ORF37 transfected cells.

(II) mitochondria functionality by JC-1, which is a cationic dye that exhibit potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green to red. As a consequence of mitochondrial depolarisation a decrease in the red/green fluorescence intensity ratio, is evidenced. JC-1 is utilized for the detection of mitochondria depolarisation occurring in the early stages of apoptosis. In control H_2O_2 treated cells there is an evident shift from red to green mitochondrial staining, indicating apoptosis (fig.28 A-B). The phenomenon is clearly less stressed in mORF37 transfected cells (fig.28 C-D).

(III) Apoptosis detection by Annexin V (data not shown) gave no clear results as the percentage of stained H_2O_2 treated cells is very low. It is instead very evident a change in cell morphology, in both transfected and control cells, and most of all, an increase in median fluorescence intensity in sham vector vs mORF37 transfected, H_2O_2 treated cells.

6. DISCUSSION

Numerous studies have illustrated the amplification and overexpression of the HER2 oncogene in human breast cancer and its association to poor clinical outcome, thus emphasizing its essential role in breast cancer pathogenesis. Because of the development of targeted therapy against HER2, and the possible role of HER2 as a predictor of chemotherapy treatment response, identification of factors that might modulate the clinical behaviour of HER2-amplified tumours has become increasingly important. Co-amplification and increased expression of several other genes adjacent to HER2 have been reported. These co-amplified and over-expressed genes might be likely candidates for factors that have an impact on the treatment responses observed in the HER2-amplified tumours. Among these, in a minimal common region of 280 Kbp, there is an open reading frame (c17-ORF37) coding for a hypothetical protein, called MGC14832.

Quantitative Real Time PCR of c17-ORF37 transcript disclosed the association with HER2 amplification in a panel of breast cancer cell lines (BT474, SKBr3, MDA MB 231 and ZR75-1) whose genetic aberrations are highly representative of those occurring in primary tumours.

However, c17-ORF37 gene product has never been reported as an overexpressed protein in tumour cells or tissues, neither is known whether it has a specific function in malignant transformation and/or in cancer development. The lack of suitable antibody, did not allow researchers to investigate on the presence of c17-ORF37 protein in malignant tissue.

To answer these questions, we first of all aimed at producing a polyclonal antibody against the N-terminal portion of the hypothetical protein, a region sharing no homology with any other known polypeptides. Despite the high specificity of the immunogen peptide used, the elicited polyclonal antibody showed, when used in western blotting of tumour cell lysates, some cross-reactivities with other proteins, making impossible its application to immunohistochemistry studies.

Nevertheless, it was an important tool to detect, for the first time, the presence of c17-ORF37 expression product in human breast cancer cells, as resulted from specific antibody reaction with a protein focusing at 12 KDa, the expected molecular

weight of MCG14832. Besides, the intensity of the staining in the different cell lysates followed the RNA levels measured by quantitative Real Time PCR.

Though the isolated cell lines, as said above, are highly representative of the *in vivo* malignant cells, they do not cover all the differences in genetic and somatic features, as, for instance, those with HER2 overexpression without gene amplification.

Actually, there is a large subset of tumours, the most difficult to treat because of their resistance to Herceptin™ therapy, that overexpress HER2 at protein level, without genetic amplification, a characteristic not borne by any isolated tumour cell lines, at least to our availability.

In order to study c17-ORF37 expression at RNA and protein level in a cell line with these features, we used as a model a mouse cell line transfected for HER2, taking advantage by the fact that in mice HER2 and c17-ORF37 are located in a strictly conserved synthenic locus (Katoh & Katoh, 2003), but gene amplification never occurs.

Unexpectedly we found that murine c17-ORF37 can be transactivated, at RNA level, by HER2, probably via PI3K/NF-kB pathway, as suggested by the dramatically decrease of c17-ORF37 transcript levels, induced by some inhibitors of this pathway.

It is well known that NF-kB is a nuclear factor activator that stimulates proliferation and blocks programmed cell death (apoptosis), in different cell types, including breast cancer, and that, among other functions, promotes transcription of genes acting as a survival factor. Furthermore its activation strongly correlates with HER2 expression (Zhou, 2000).

In an attempt to find a role to c17-ORF37 gene product, knock-down experiments were performed by Dr. Kallioniemi's group at the University of Tampere, Finland: silencing c17-ORF37 gene in human cell line, with a specific si-RNA didn't cause grave modification to cell viability, morphology and overall metabolisms (personal communication).

To the same purpose, we followed the opposite way, that is, we tempted "gain of function" experiments by transfecting mouse fibroblasts with mouse c17-ORF37 gene (mORF37), under the control of a strong promoter such as CMV.

Transfected cells did not modify their morphology, neither changed overall viability or growth ability, in respect to control cells.

To test the hypothesis that the protein acts as a survival factor, as suggested by PI3K/NF-kB involvement, and calling to mind that MG14832 *in silico* structure predicts it to be a Seleno-protein (Seleno-proteins are a class of protein controlling the redox status of the cell), mORF37 transfected cells were submitted to an exogenous potent oxidative insult, such as the one given by H_2O_2.

The results, while confirming that cells subjected to hexogen H_2O_2, die from both apoptosis and necrosis (Kannan, 2000; Mao, 2006), show that these cells acquired resistance capacity to this oxidative stress as evidenced by toxicity assay and by microscopic analysis. Nuclei from mORF37 transfected cells appeared less damaged in respect to control cells, as well as the mitochondrial potential less altered. These observations suggest the idea that MG14832 might exert its protection on apoptosis pathway, at least for short time exposition of the oxidant.

To understand whether MG14832 exerts its protection by acting on either the apoptosis or necrosis pathway, or on both of them, we made some other experiments, like Annexin V binding.

The data collected did not allow the answer: at least at low doses of H_2O_2, a weak Annexin V binding, both on transfected and control cells (data not shown), together with a change in cell morphology, was detected from FACS analysis.

On one hand, this altered morphology may reflect the lost of selective membrane permeability and increased rigidity, as already reported for cells exposed to H_2O_2 stress (Davies, 2000): this may account for the very low binding of Annexin V that would subtract to the detection, cells eventually engaged in apoptosis. However, the few positively stained cells showed a higher median fluorescence intensity in the control cells than in the transfected ones, possibly indicating the latter as less prone to apoptosis. On the other hand, the altered morphology of the cells could simply be an expression of the ongoing necrosis process, which is evidenced by the release of LDH when higher concentration of H_2O_2 were used for longer time.

Data are then not explicative or conclusive about which pathway MG14832 might act on. Moreover, the possibility of an anti-apoptotic activity induced through dead factor receptors family (FAS, TNF) should be investigated. HER2 transfected mouse fibroblast are resistant to TNF-α (Zhou, 2000) via the Akt/NF-kB pathway, witch is

constitutively activated, so the involvement of MGC14832, that, from our results, seems to be activated from the same pathway, has to be taken into account.

As regards the search for a specific enzymatic activity of MGC14832 that may explain the protection from peroxides, a set of experiments have been conducted to detect some peroxidase activities, as it might be supposed from its *in silico* structure. All the attempts made, failed, for the reasons hypothesized in the experimental section.

By now we can only suppose MGC14832 as a survival factor, overexpressed by either gene amplification or by activated PI3K/NF-kB pathway, acting on both apoptosis and necrosis.

From several lines of evidence, from both human cancer and animal models, Weinberg (Hanahan and Weinberg, 2000) proposed that few (six) alterations in cell physiology are essential to collectively dictate malignant transformation and growth, and that tumour development proceeds as a Darwinian evolutionary process in which a succession of genetic changes lead to a progressive conversion of normal cell into cancer cell.

In the case of HER2 amplicon, there is a concomitant gain of pro-tumour functions, given from genes not only co-amplified, but also overexpressed (Kauraniemi, 2003): self sufficiency in growth signals (HER2), increased proliferation from hormone action (MLN64 facilitates steroid hormone production in cancer cells), (Akiyama, 1997), metastasis properties (GRB7 codes for a SH2 domain containing growth factor receptor tyrosine kinase that has a function in cell migration), (Shen, 2002) and, from our data, resistance to oxidative attack, probably through evasion from programmed cell death (MGC14832). Besides, this last function might be acquired together with HER2 overexpression even in the absence of gene amplification.

What is the significance of the connection of HER2 overexpression, generated either from genetic or epigenetic, with MGC14832? Is it specific of c17-ORF37, or may it also concern some other genes of the amplicon? Is there some particular cellular program that links together HER2 and c17-ORF37 overexpression, irrespectively of their gene amplification, or vice versa they do not respond to the same program? Can we find from the answer to these questions an explanation on why Herceptin™ only works with amplified overexpressing HER2 tumours?

The finding that its expression is not strictly depending on the level of gene amplification, enforce the possibility that measuring MG14832 expression in HER2 positive tumour tissues (amplified or not), may add information on the characteristics of the tumour itself, in term of both prognosis and therapeutic indication.

There is a need for a monoclonal antibody that, in collaboration with Genesis Biotech Inc., is under production while this thesis is on printing, to detect MGC14832 on fresh or fixed breast cancer tissue, and eventually to make an array on the *in vivo* distribution of the protein.

In addition, the monoclonal antibody may allow the isolation and purification of the protein in its native form, that could be essential to discover its enzymatic activity, if any.

7. FIGURES

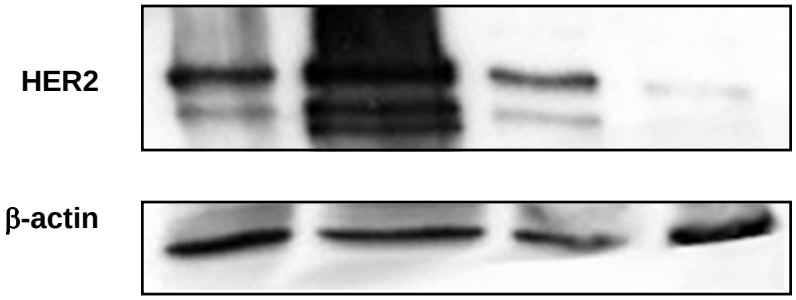


Fig.1: Western Blotting analysis. 50 μg of whole cell lysates were run on SDS PAGE. Proteins transferred to nitrocellulose membrane were incubated with C-18 (Santa Cruz), a polyclonal anti HER2 antibody, and re-incubated after stripping with anti β-actin monoclonal antibody (Sigma).

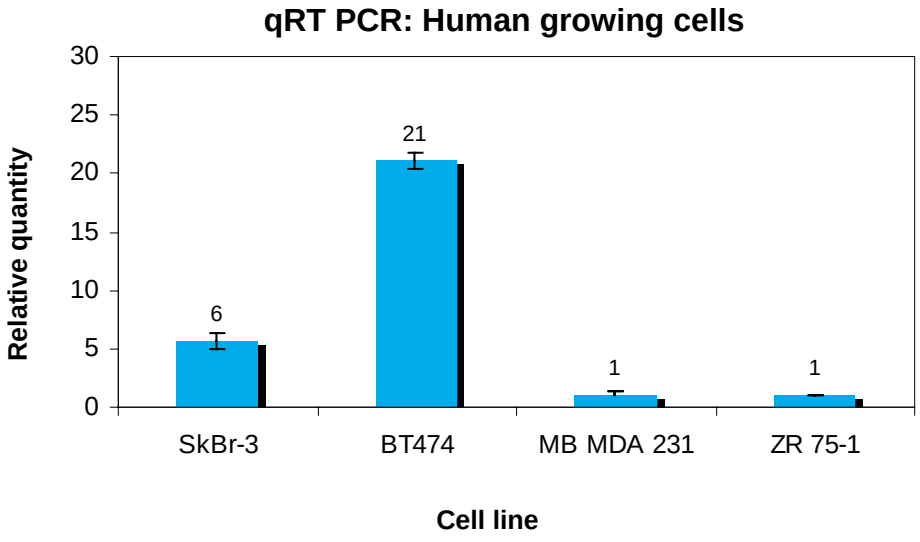


Fig.2 :Quantitative Real Time PCR. c17-ORF37 mRNA relative quantity on human cell lines with different HER2 status. Cells were grown in complete medium

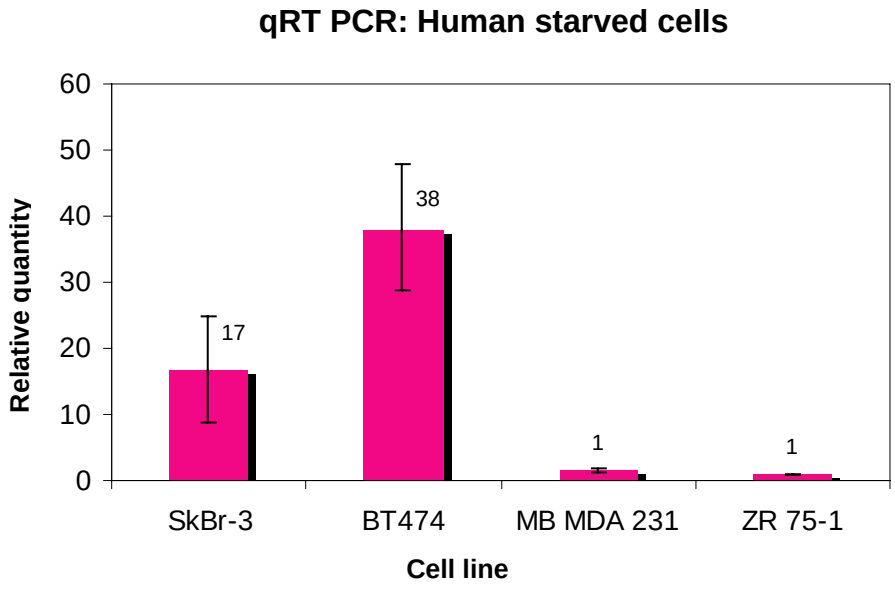


Fig.3: **Quantitative Real Time PCR.** c17-ORF37 mRNA relative quantity on human cell lines with different HER2 status. Cells were grown in medium without serum (starving) .

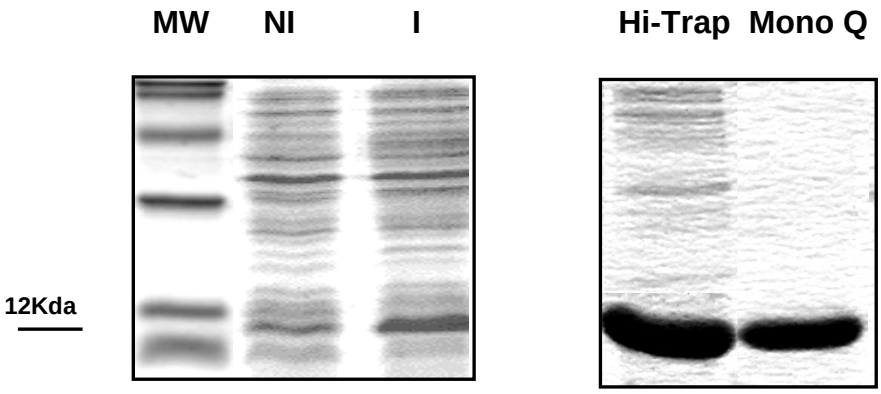


Fig.4: SDS-PAGE of recombinant protein recORF37. Induction, (first panel) and purification (second panel) of recORF37 recombinant protein . The band of induced culture (I) is strongly visible at 12 Kda, while is absent in the not induced (NI) one. Protein was first purified by using an Hi-Trap Chelating column (Amersham), and then a Mono-Q column, in FPLC chromatography apparatus.

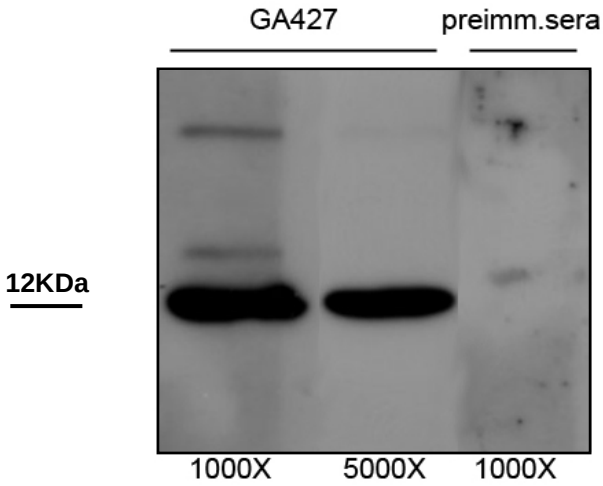


Fig.5: Western Blotting analysis . 3μg of recORF37 recombinant protein were run on SDS PAGE and proteins transferred to nitrocellulose membrane were incubated with indicated dilution of GA427, or preimmune rabbit sera.

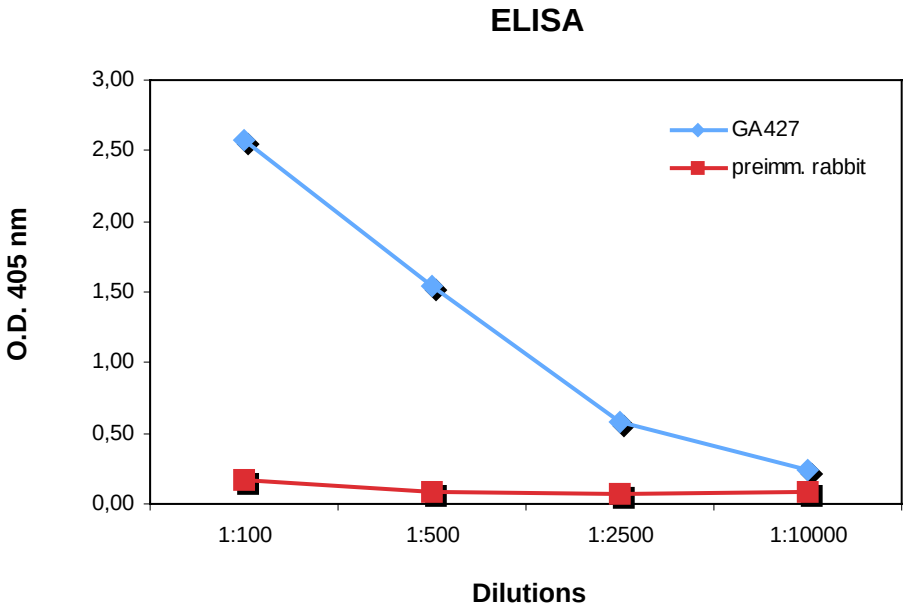


Fig.6: ELISA. 100ng of recORF37 recombinant protein were coated on the plate and incubated with the indicated dilution of GA427 or preimmune rabbit sera.

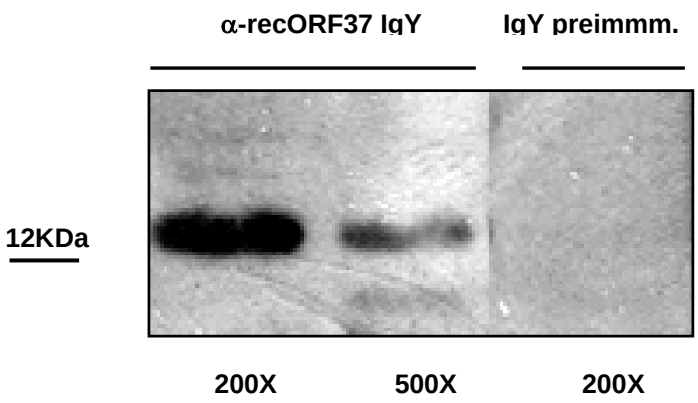


Fig.7: Western Blotting analysis. 3μg of recORF37 recombinant protein were run on SDS PAGE and proteins transferred to nitrocellulose membrane were incubated with indicated dilution of α-recORF37 IgY, or preimmune IgY.

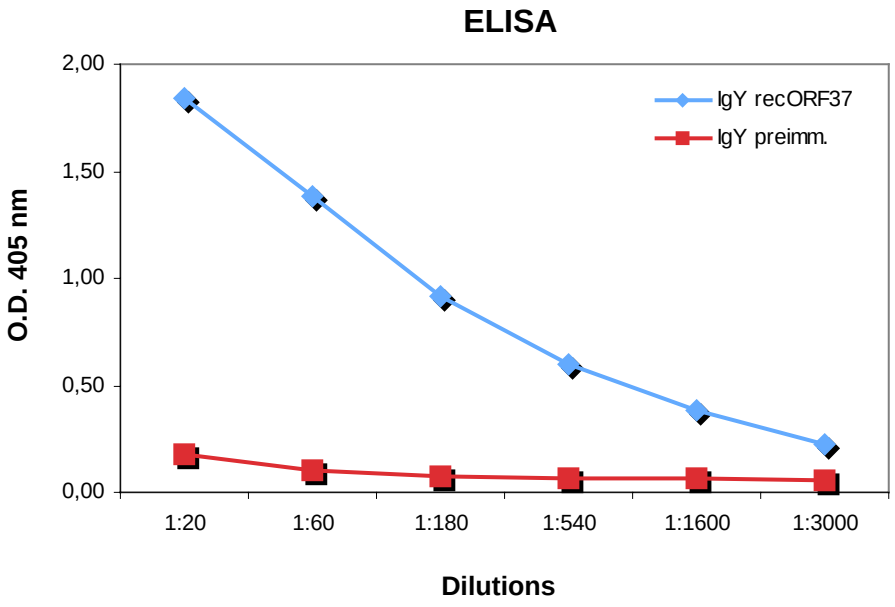


Fig.8: ELISA. 100ng of recORF37 recombinant protein were coated on the plate and incubated with the indicated dilution of α-recORF37 IgY, or preimmune IgY.

1 EPGQTSVAPPPEEVEPGSG 19	synthetic peptide
4 EPGQTSVAPPPEEVEPGSG 22	Homo Sapiens Identities = 19/19 (100%), Positives = 19/19 (100%), Gaps = 0/19 (0%)

Table A: Human 4-22 Synthetic Peptide: The synthetic peptide encompassing the amminoacidic sequence 4-22 of Human MGC14832 is the protein portion sharing no similarity with any other human protein.

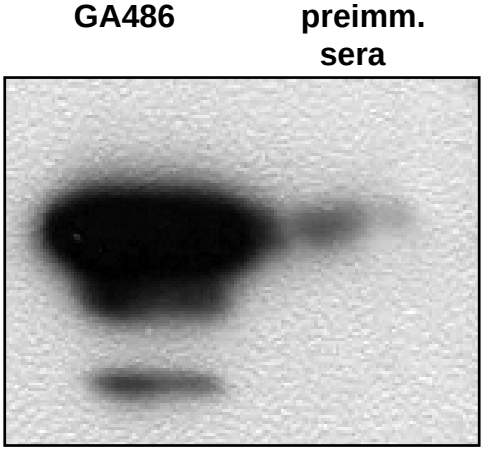


Fig.9: Western Blotting analysis. 3μg of 4-22aa hORF37 epitope peptide (fused to KLH) were run on SDS PAGE. Proteins were transferred to nitrocellulose membrane and incubated (1:500) with GA486 polyclonal antibody or preimmune rabbit sera.

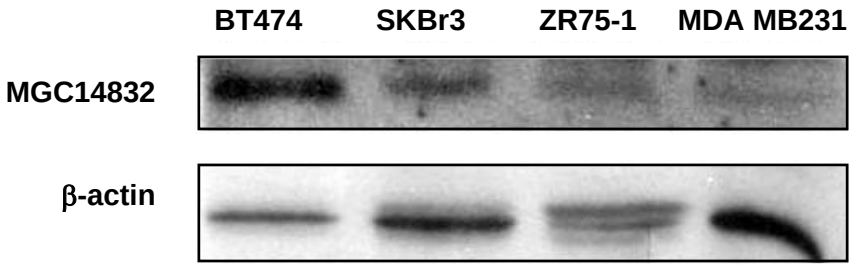


Fig.10: Western Blotting analysis. 50 μg of whole cell lysates were run on SDS PAGE. Proteins transferred to nitrocellulose membrane were incubated with GA486, and re-incubated after stripping with anti β-actin monoclonal antibody (Sigma).

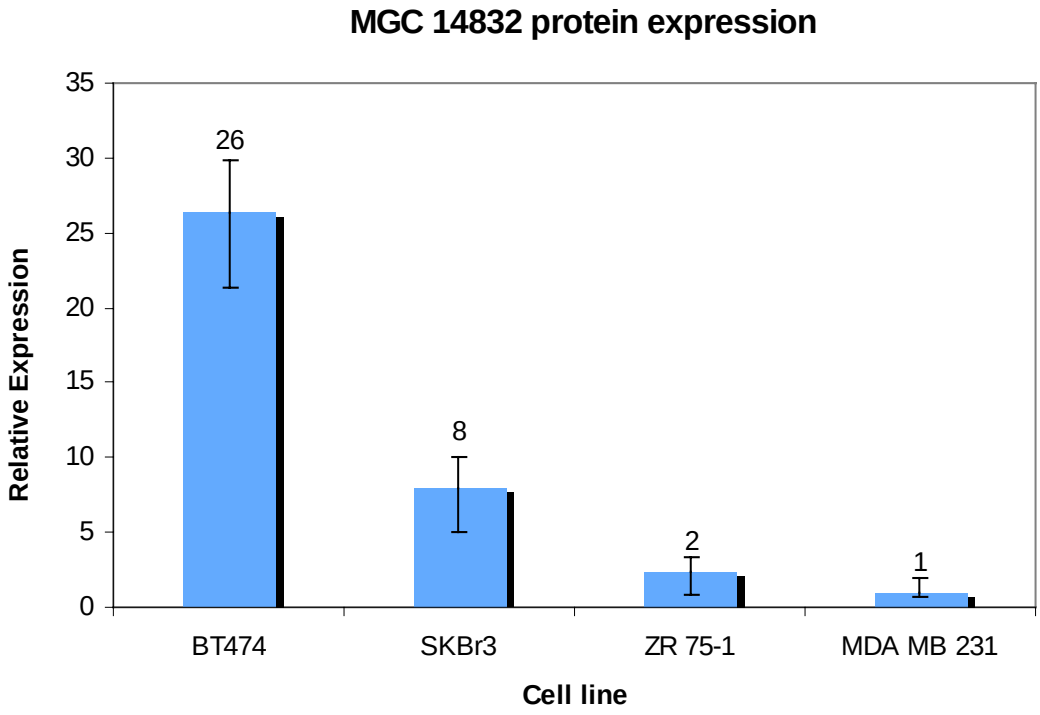


Fig.11: Densitometric scan of WB bands. Bands from fig.10 were quantified by an imaging densitometer (GS-670, Biorad). Relative quantities of MGC14832, normalized to the housekeeping protein β-actin, were reported in respect to MDA MB 231, chosen as basally expressing cells.

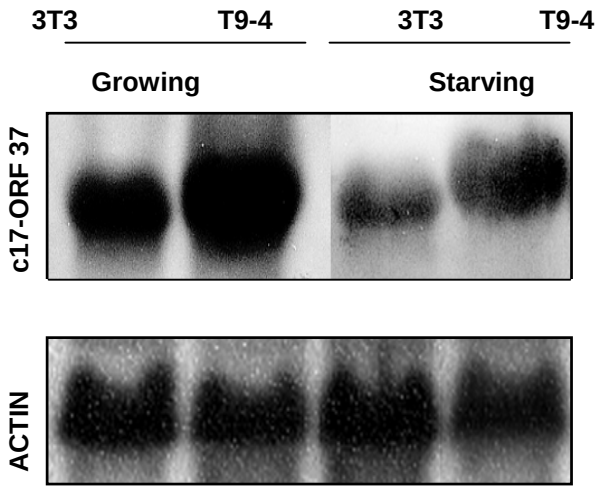


Fig.12: Northern Blotting analysis. 15 μg of total RNA from T9-4 and NIH 3T3 (grown in complete or starving medium). Hybridisation was performed at 63°C either with mORF37 or β-actin specific probes.

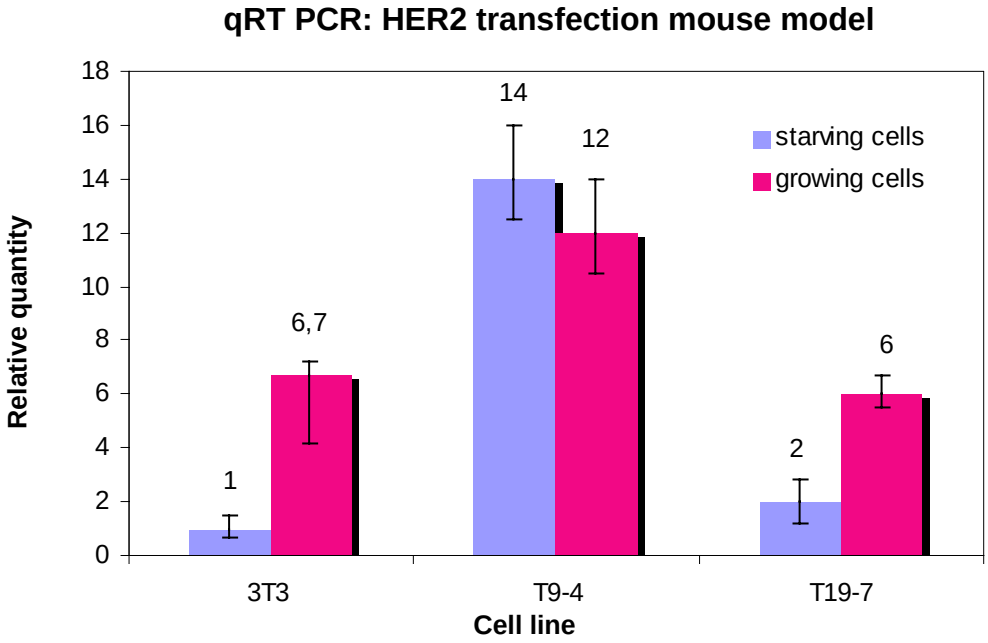


Fig.13: **Quantitative Real Time PCR.** c17-ORF37 mRNA relative quantity on mouse cell line model. Cells were grown in complete medium or medium without serum (starving). Data were calibrated on NIH 3T3 grown in starving medium. (T9-4: HER2 transfected NIH3T3, T19-7:HER1/HER2 chimera transfected NIH3T3)

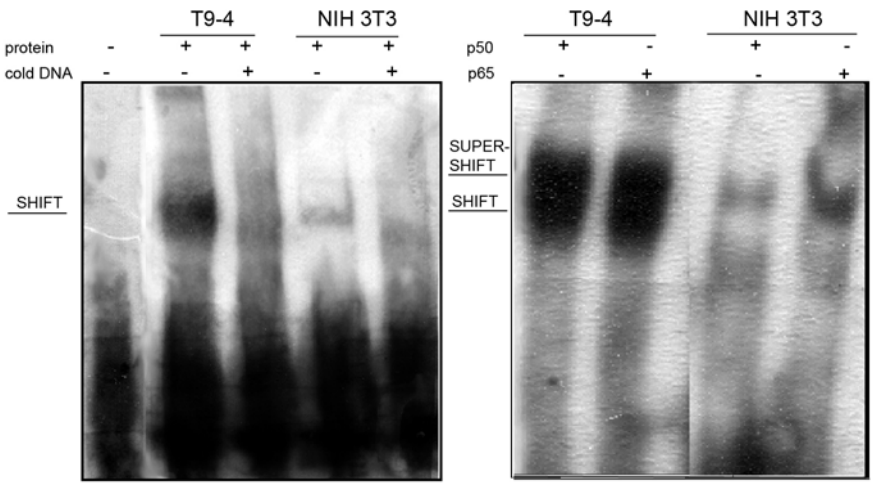


Fig.14 Electromobility shift assay (EMSA): 5 μg of nuclear proteins of starving cells. Run was performed with double stranded DNA corresponding to NF-kB binding site, with or without (cold DNA) biotinilation (Shift) and with antibodies against each NF-kB subunit (p50, p65) (Supershift).

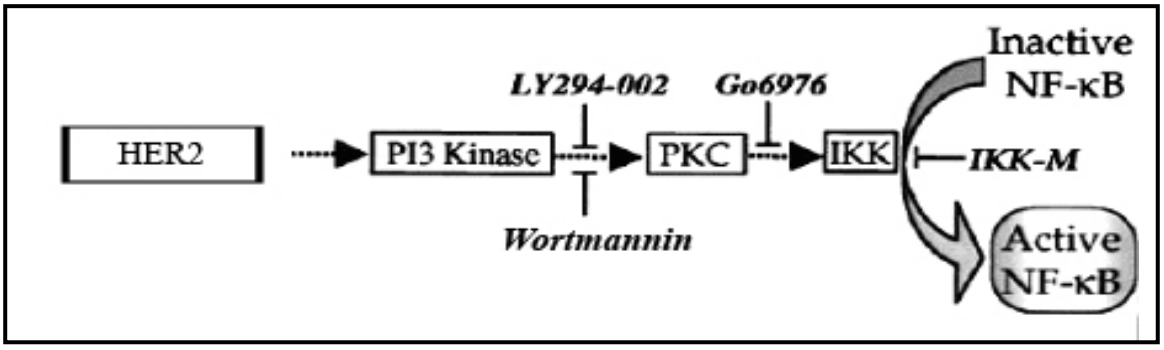


Fig. 15. HER2/PI3K/NF-kB pathway and inhibitors: 1) Wortmannin is an irreversible inhibitor of PI3K that doesn't affect upstream signal events (PI4K is inhibited at a concentration 100-fold higher).
2) Ly294-002 is a PI3K inhibitor that acts on the ATP binding site of the enzyme, not affecting EGFR, PKC, or PI4K activity.
3) Go6976 is an inhibitor of Ca^{++} dependent PKC.

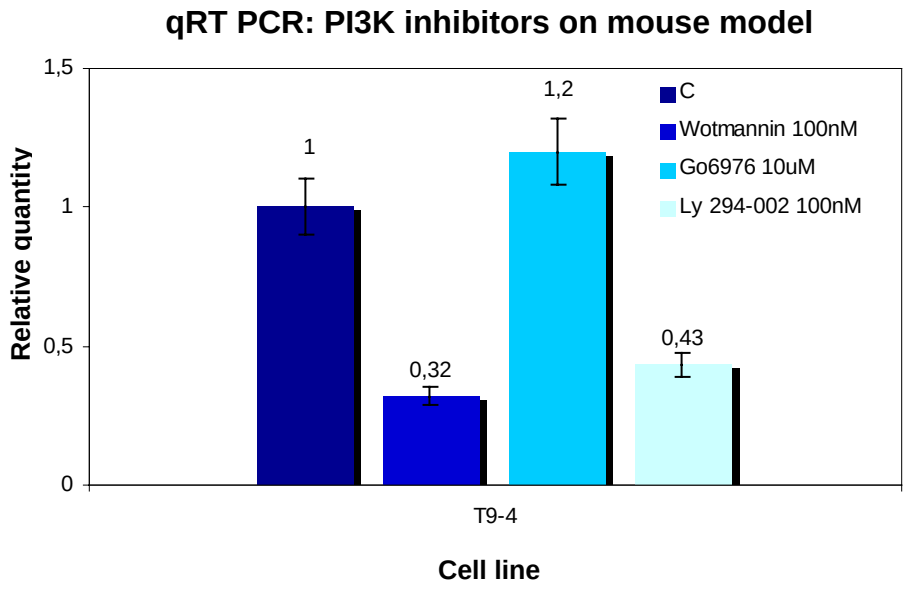


Fig 16: Quantitative Real Time PCR. c17-ORF37 mRNA relative quantity on mouse cell line model. Cells were treated for 24h in starving medium with Wortmannin (100nM) or Go6976 (10μM) or Ly294-002 (100nM). c17-ORF37 transcript is calibrated vs untreated cells (C).

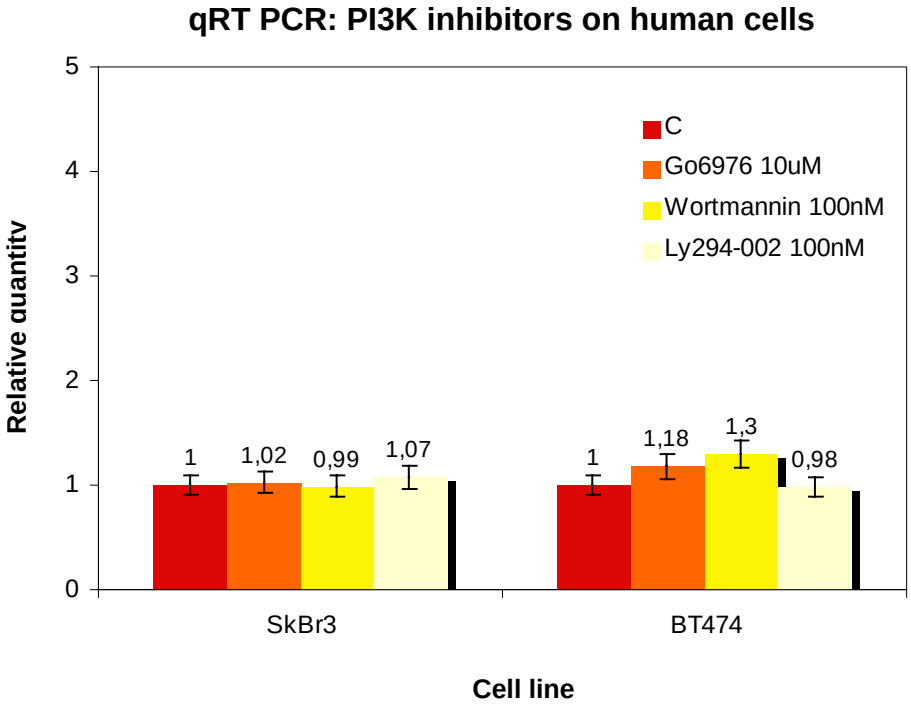


Fig 17: Quantitative Real Time PCR. c17-ORF37 mRNA relative quantity on BT474 and SkBr3 human cell lines. Cells were treated for 24h in starving medium with Wortmannin (100nM) or Go6976 (10μM) or Ly294-002 (100nM). c17-ORF37 transcript is calibrated vs untreated cells (C).

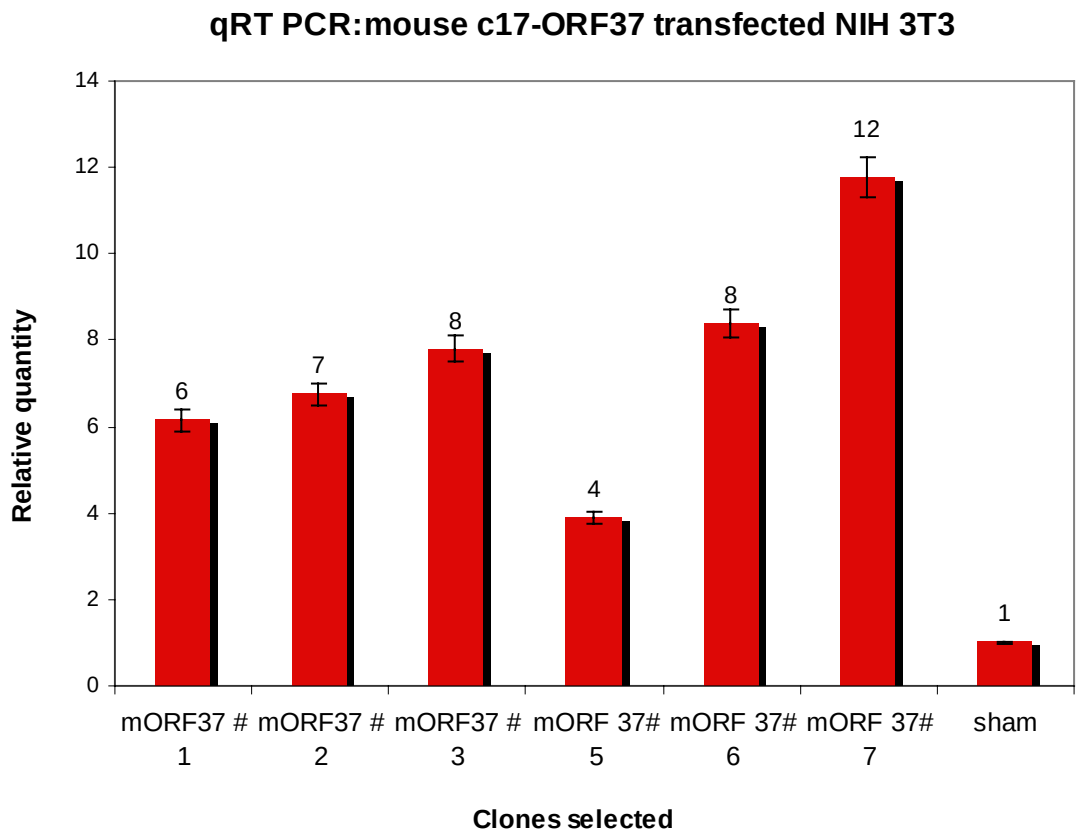


Fig.18: Quantitative Real Time PCR. c17-ORF37 mRNA relative quantity of mORF37 transfected vs. empty vector transfected NIH 3T3. Cells were grown in medium without serum (starving).

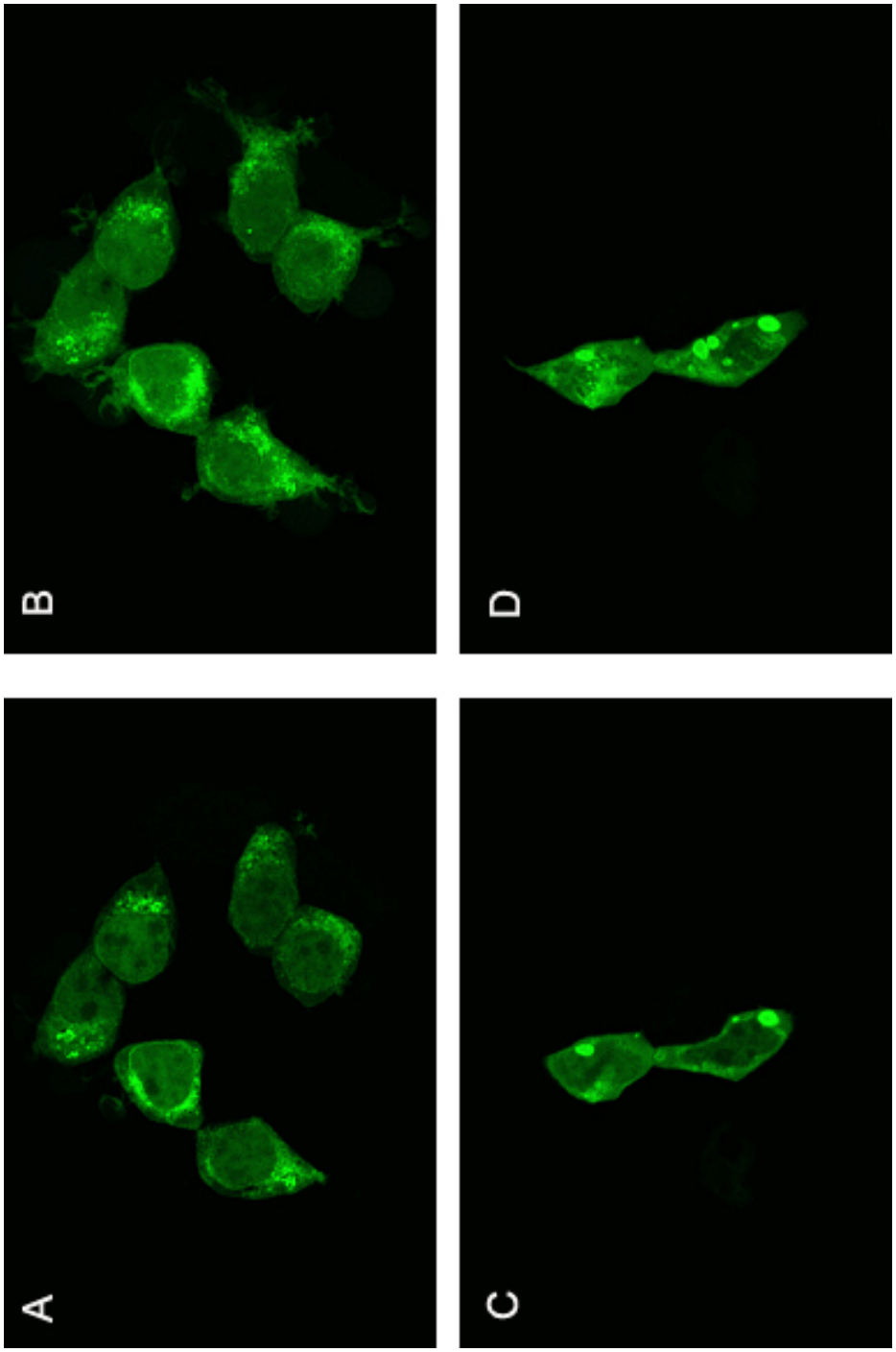


Fig. 19: Confocal laser microscopy of GFP-mORF37 transfected cells. The distribution of fluorescence inside the cell appears in a well characterised fashion, and seems not to be casually widespread in the cytoplasm. It gives the idea of a clear internal distribution, with a “donuts like” morphology, mostly perimembrane (A: central optical section, B: z axis reconstruction). In some cases the fluorescence is very intense, condensed in some “vesicle like” structure (C: central optical section, D: z axis reconstruction).

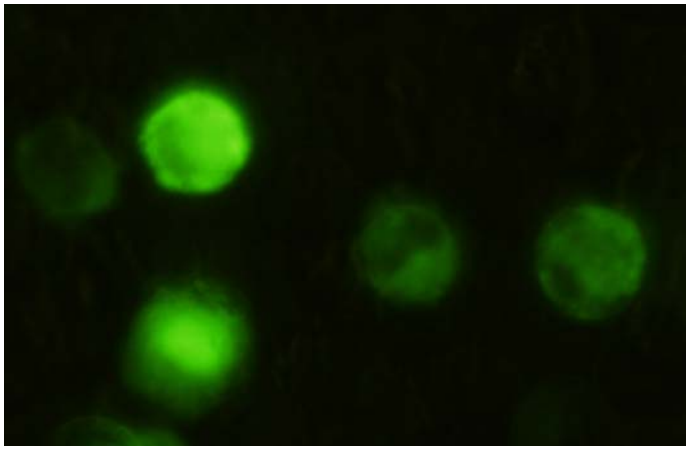


Fig. 20: Confocal laser microscopy of pGFP transfected cells. The distribution of the fluorescence is casual and widespread in all the cell.

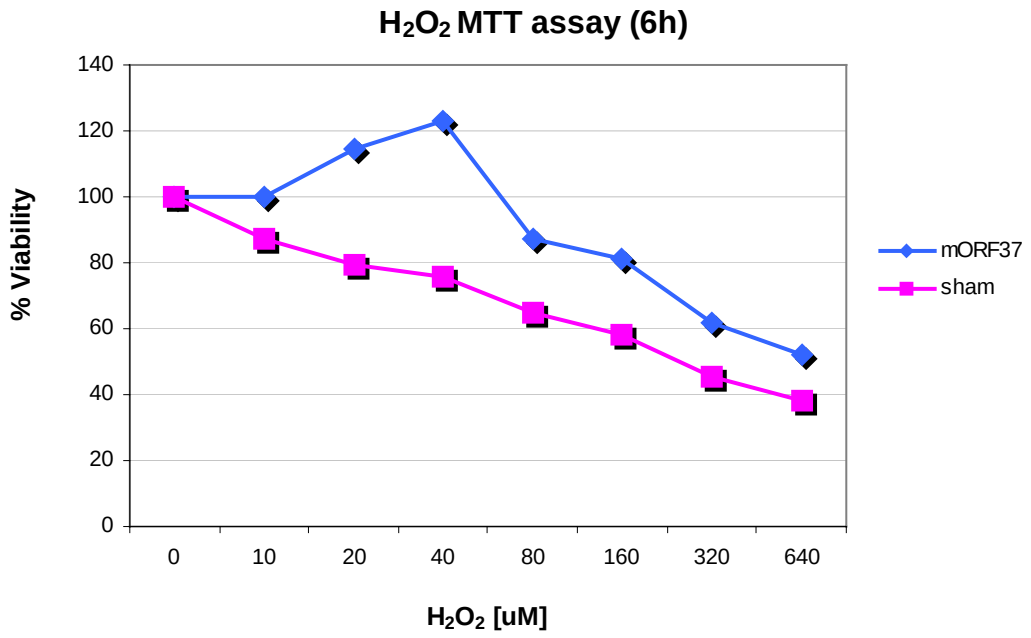


Fig.21: H_2O_2 MTT assay. $2x10^4$ transfected NIH 3T3 were plated on a 96 wells plate and treated with H_2O_2 for 6h at the indicated dilutions. Viability was evaluated by MTT assay.

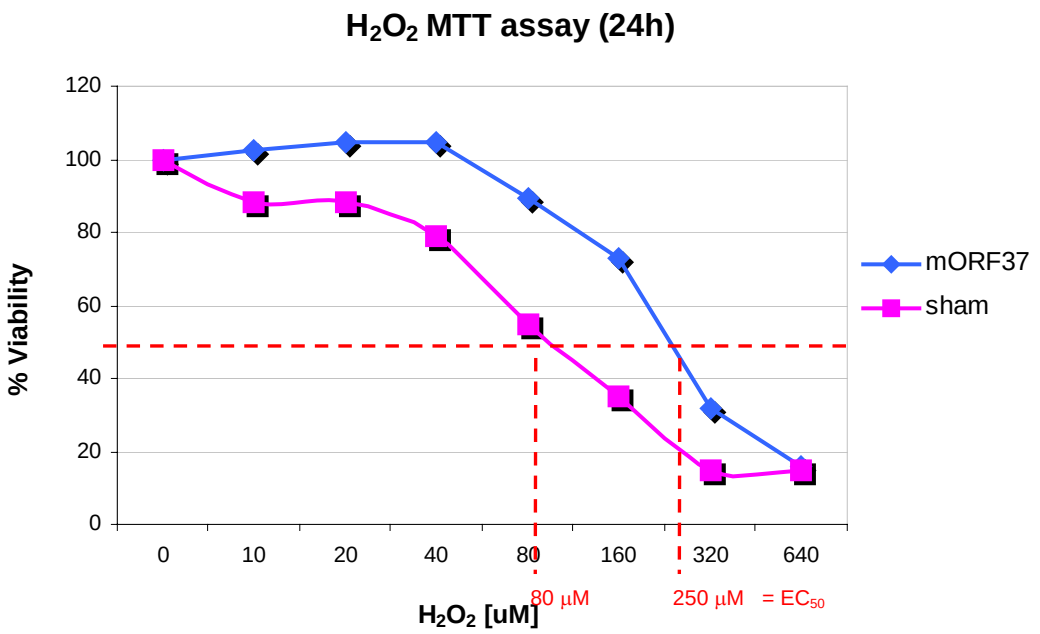


Fig.22: H_2O_2 toxicity assay. 2x10^4 transfected NIH 3T3 were plated on a 96 wells plate and treated H_2O_2 for 24h at the indicated dilution. Viability was evaluated by MTT assay. H_2O_2 effective dose at 50% (EC_{50}) was 250 or 80 µM for mORF37 transfected or sham vector transfected NIH 3T3, respectively.

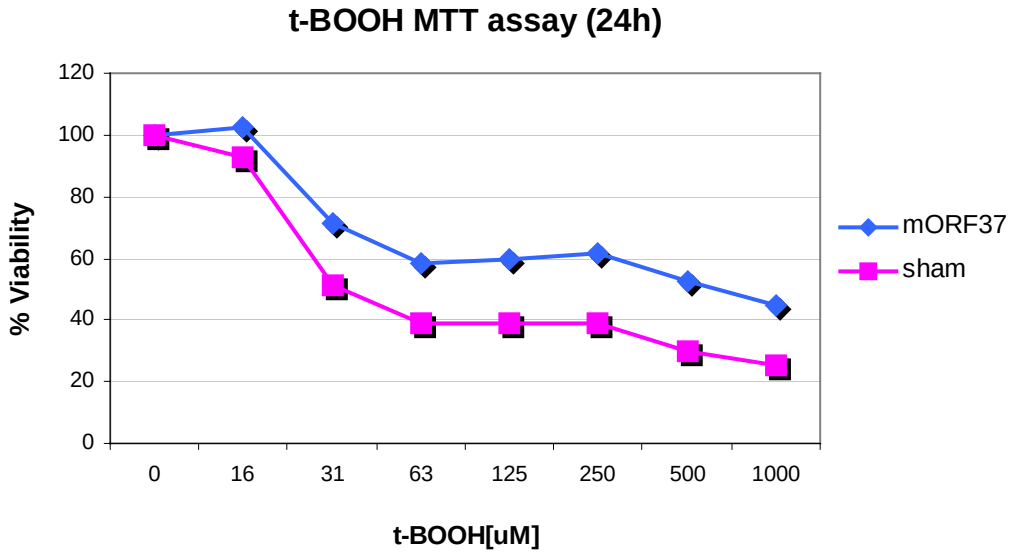


Fig.23: t-BOOH MTT assay. 2x10^4 transfected NIH 3T3 were plated on a 96 wells plate and treated with indicated dilution of tert-butyl-hydroperoxide, (t-BOOH), for 24h. Viability was evaluated by MTT assay. t-BOOH effective dose at 50% (EC_{50}) was 250 or 30 µM for mORF37 transfected or sham vector transfected NIH 3T3, respectively.

1)

	EC_{50}	
	Sham	mORF37
H_2O_2	80 uM	250 uM
t-BOOH	30 uM	250 uM

2)

	NIH 3T3 transfected		
	Sham	mORF37	recORF37
GSH Px	-	-	-
Luminol Px	-	-	-
Catalase-like	-	-	-

(-) = negative

Table B: First panel: Summary of EC_{50} measured on transfected NIH 3T3 for both H_2O_2 and t-BOOH. **Second panel:** Peroxidase activity assays performed. All tests gave negative results, both on transfected cells and on recombinant protein, recORF37.

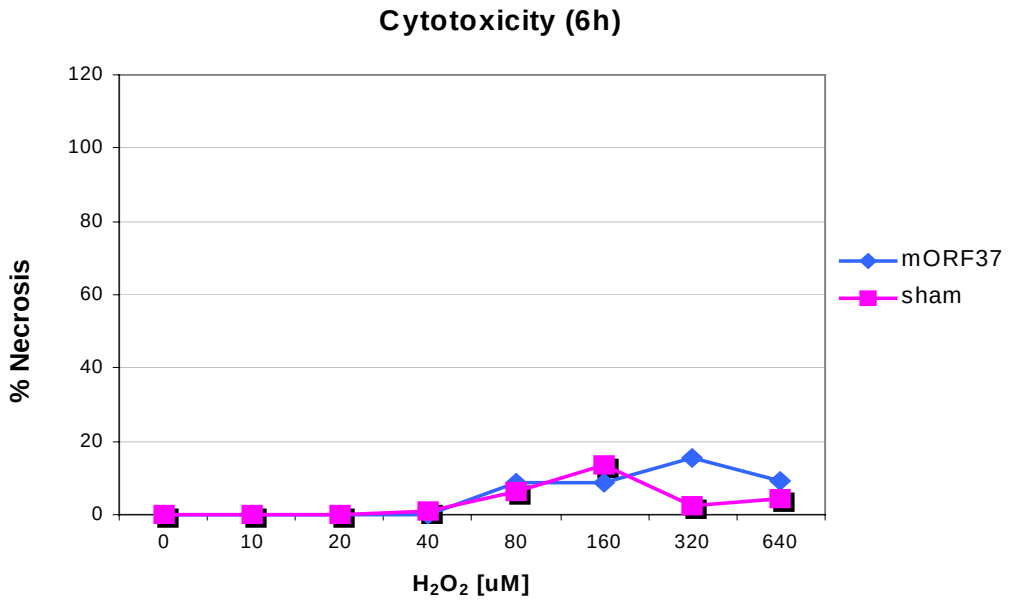


Fig.24: H_2O_2 cytotoxicity assay. $2x10^4$ transfected NIH 3T3 were plated on a 96 wells plate and treated with H_2O_2 for 6h at the indicated dilutions. Necrosis was evaluated on 50 μl of supernatant measuring LDH production.

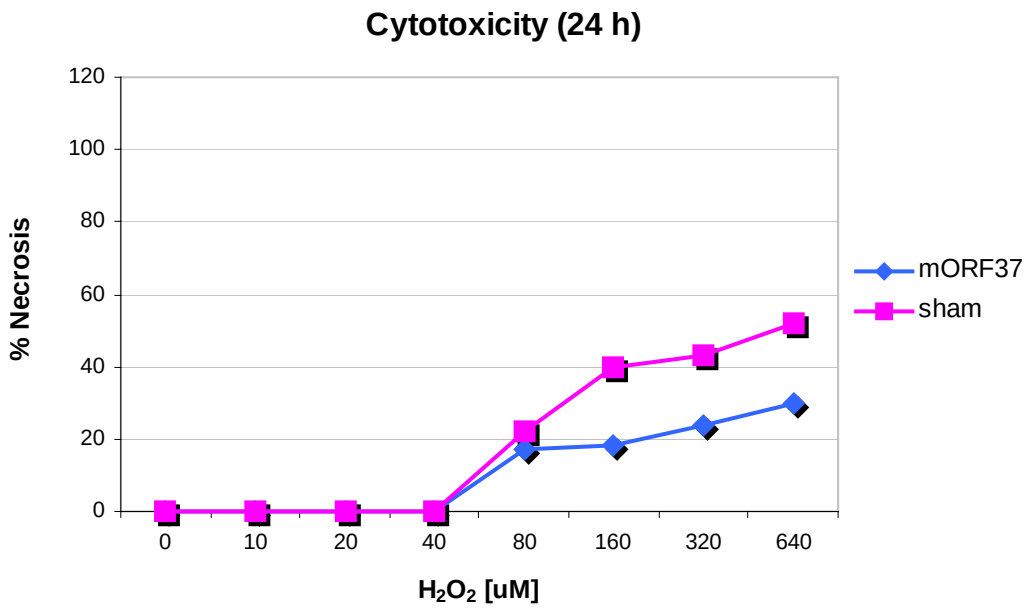


Fig.25: H_2O_2 cytotoxicity assay. $2x10^4$ transfected NIH 3T3 were plated on a 96 wells plate and treated with H_2O_2 for 24h at the indicated dilutions. Necrosis was evaluated on 50 μl of supernatant measuring LDH production .

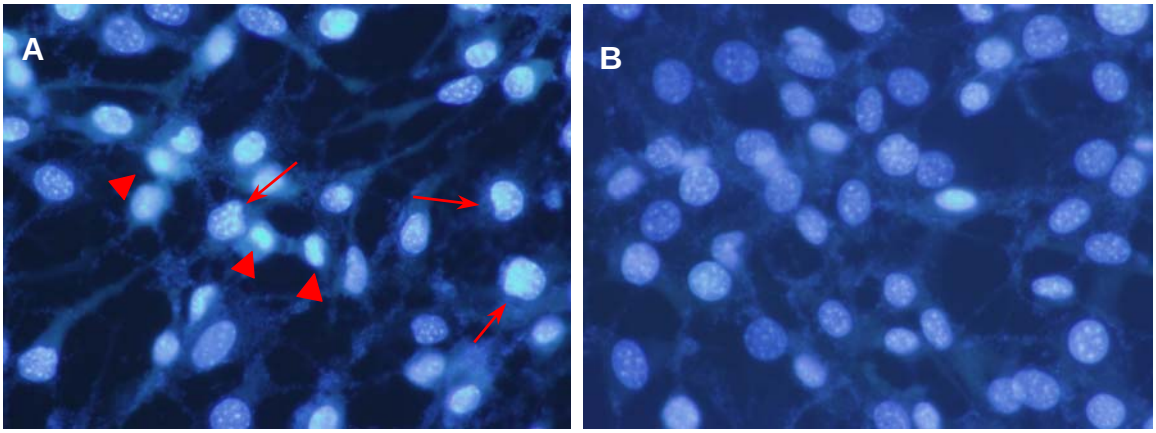


Fig. 26: Hoechst 33258 staining of empty vector transfected cells. A: Sham vector transfected cells treated with 300 μM H_2O_2 for 2 h presents nuclear condensation (arrowheads), and blebbing (arrow), and a decrease in nucleus/cytoplasm ratio, in comparison with untreated cells (**B**).

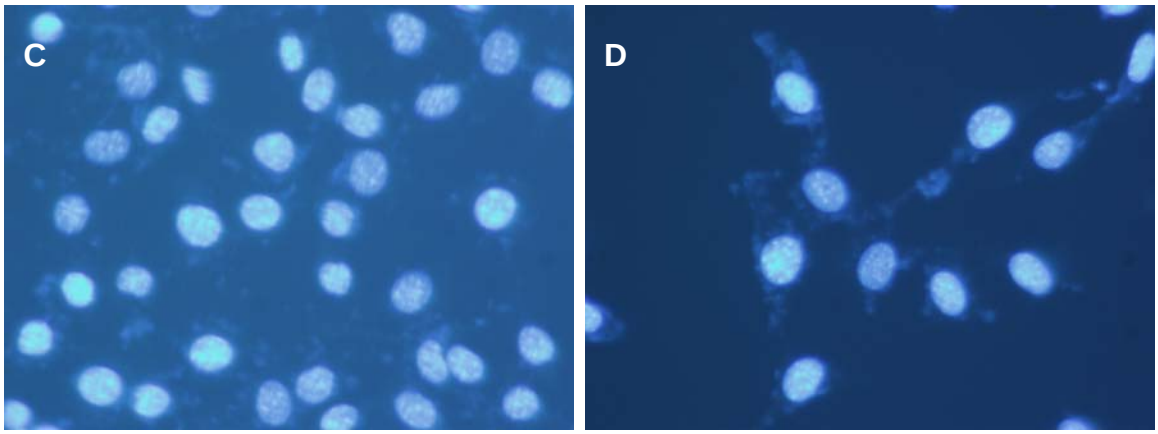


Fig. 27: Hoechst 33258 staining of mORF37 vector transfected cells. C: mORF37 vector transfected cells treated with 300 μM H_2O_2 for 2 h doesn't presents nuclear condensation or blebbing, and nucleus/cytoplasm ratios is unaltered. **D:** Untreated cells .

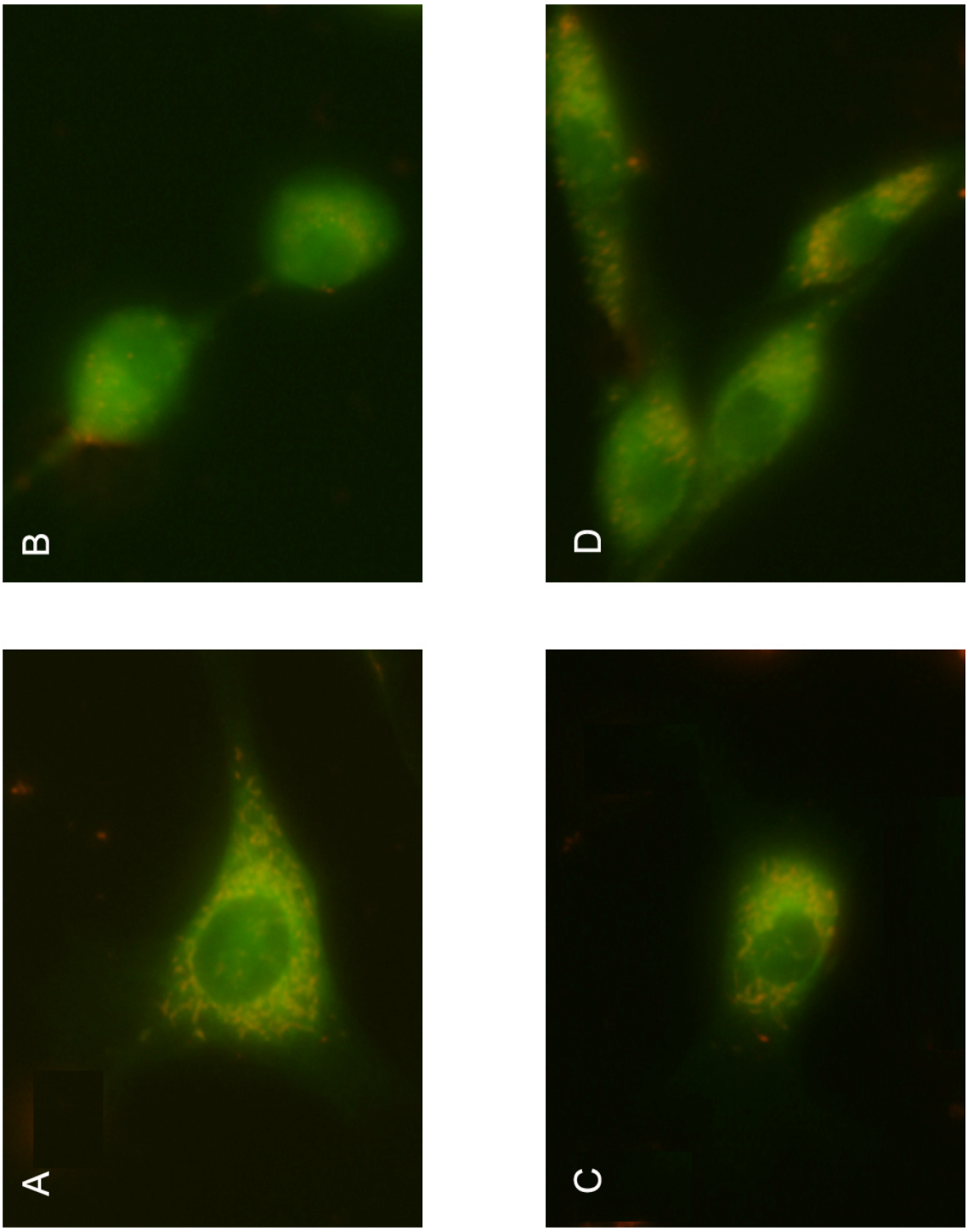


Fig. 28: JC-1 staining of control or mORF37 vector transfected cells. Empty vector transfected cells treated (B) with 300 μM H_2O_2 for 2 h presents a great decrease in red/green fluorescence intensity ratio in respect to untreated cells (A). The phenomenon, indicating an early stage of apoptosis, is less stressed in mORF37 transfected cells (C: untreated, D: treated with 300 μM H_2O_2 for 2 h).

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