Study of position effect as a mechanism arising from chromosomal translocations in leukaemia

A thesis submitted for the degree of Master of Philosophy

By

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Abstract

The chromosomal translocation of t(14;18)(14q32;18q21) is a characteristic aberration of follicular lymphoma and Diffuse Large B cells lymphoma. By PCR, it was proved that the rearrangement of chromosomes 14 and 18 leads to an overexpression of *BCL2*, an anti-apoptotic protein, which is one of the factors responsible for the maturation of the diseases. The translocation involves the promoter region of *IGH* gene and the transcriptional unit of *BCL2* gene.

Previous studies carried out in Dr Tosi's lab showed a looping out of the *BCL2* gene from its chromosome territory in 15% of the nuclei analysed. This looping out could be possibly responsible for the transcriptional activity of the gene. A further relevant finding concerns the spatial distribution of the genes involved in the translocation in the interphase nuclei. In the Pfeiffer cell line, harbouring the t(14;18) rearrangement, the translocated *BCL2* gene was positioned in the cell nuclei according to a bimodal distribution. One could speculate that the distribution in the periphery and in the centre of the nuclei could divide the Pfeiffer cell line in two different subpopulations, consequently from the transcriptional activity.

These preliminary data set the ground for more experimental work to test whether genes associated with the nuclear interior were transcriptionally active as opposed to the genes positioned towards the nuclear periphery, transcriptionally inactive. The work here presented focuses on this investigation using RNA-DNA FISH (Fluorescence in situ hybridization). My work enabled the detection of *IGH*, *BCL2* and t(14;18) genes along with their transcripts inside of the nuclei of Pfeiffer cell line. Contrary to what had been hinted by previous work, my results showed multiple nuclear positions of transcriptionally active *IGH/BCL2* translocation. The result will need to be further supported by software analysis in order to define its specific nuclear position and to ensure the perfect localization of the genes inside each nucleus.

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Acknowledgements

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Chapter 1

INTRODUCTION

1.1 Lymphomas and their origin

Our body is crossed by a net of lymphatic vessels which carries draining fluid to all the tissues and organs; this is known as the lymph. This is a nutritive substance that contains metabolic products, lymphocytes, but even pathogens and foreign molecules. To filter the lymph, there are around 500 lymph nodes, which clean up the fluid in many different sites of the body. There are also some organs that are included in the lymphatic system, as spleen and tonsils. Furthermore, the lymphocytes act as protectors from infections, pathogens and any outsider attacks, along with the other components of immune system.

The lymphocytes are divided in T and B cells, both are components of adaptive immunity. The former originate from bone marrow and mature in the thymus gland; they provide supportive to B cells and can directly respond to foreign attacks. The B cells originate in the bone marrow, but mature in lymph nodes. They contribute to the immune response by the production of antibodies.
Sometimes the adaptive immune system cells gives rise to malignant forms leading to insurgence of lymphomas, consequently to causes barely identified. In other words, the lymphomas are the solid tumours of the immune system. This can affect lymphatic tissues and organs, but also other ones as brain.

The overall classification of lymphomas is: Hodgkin and non-Hodgkin. The name originates from the first clinician who catalogued under the same pathology the symptoms from different patients. He lived in the first half of 19th century and his name was Thomas Hodgkin, curator of Guy's Hospital. Only after hundred and fifty years later, the importance of his findings was recognized (Geller, 1984).
The cases attributable to Hodgkin's lymphomas are few compared to non-Hodgkin lymphomas. They start generally from T cells and affect only the lymph nodes.
The others lymphomas are included in the non-Hodgkin's disease group, which is subdivided in several subgroups linked to the origin of the transformed cells. It can affect many different organs and they are discernible during the degeneration of the disease.
The non-Hodgkin lymphomas make up around 40% of all the cases of lymphomas, but the causes are different for every subgroup (Shankland, et al., 2012). One of the most popular lymphomas is the diffuse large B-cells lymphoma (DLBCL). It is

found in 30% of new cases in USA and it's characterized by the translocation t(14;18) (14q32;18q21) (Vega & Medeiros, 2003).

One of the principal reasons of insurgence of lymphomas is related to the chromosomal translocations. They are aberrations of two different chromosomal regions that bring to the formation of a new transcriptional unit or an overexpression of oncogene.. In addition, some chromosomal translocations can be typically found in healthy individuals, as the rearrangement between *IgH* and *BCL2* gene (Janz, et al., 2003). The importance of chromosomal translocations starts from the findings that they are found in precancerous conditions and then these cells may transform and develop malignancies (Nishida, et al., 1997). Progression towards malignancy is usually due to the presence of additional genetic abnormalities such as gene mutations or chromosomal rearrangements (Griffiths, et al., 2000).

There are prevalent mechanisms resulting from chromosomal translocations in non-Hodgkin's lymphoma: the first one is when two genes are disrupted and they are then bound in the same transcriptional unit resulting in the production of a chimeric protein. One recurrent reciprocal balanced translocation is the t(2;5), which leads to the production of a new protein ALK-NPM. This is found in the anaplastic large-cell lymphoma arising from T-cell activated. The genes involved are ALK coding for a tyrosine kinase receptor and NPM coding a nucleolar phosphoprotein (Vega F., 2003).
The second mechanism is characterized by an oncogene or coding unit that moves next to another gene, usually identified with an antigen receptor gene. One of the most studied examples is the t(14;18) involving the *BCL2* gene, coding for an anti-apoptotic protein and *IgH*, immunoglobulin heavy chain region. The specific translocation is one of the features of follicular lymphoma and it will be discussed more thoroughly below.
The overexpression of oncogene due to a chromosomal rearrangement is the most common cause of B-cell cancers. They can be produced in different stages during the formation of B-cells and the overall reason is due to a switch of promoter (Robbiani DF and Nussenzweig MC, 2013).

The specific mechanism of formation of chromosomal translocations allows their cataloguing. They are classified into Reciprocal and Robertsonian translocations

(Figure 1). The first one is caused by a swap of two regions from non-homologous chromosomes during the rearrangement. The translocation is described as balanced when there is not loss or gain of genetic material and unbalanced when the swap causes loss or gain of genetic information. The second type of translocation is defined as Robertsonian, from the name of the American biologist who discovered them. They can involve only the five acrocentric chromosomes (13, 14, 15, 21 and 22) and they can join parts of non-homologues or homologues chromosomes. The result of these last rearrangements is one chromosome with long arm and the loss of the short arm during cell cycle.

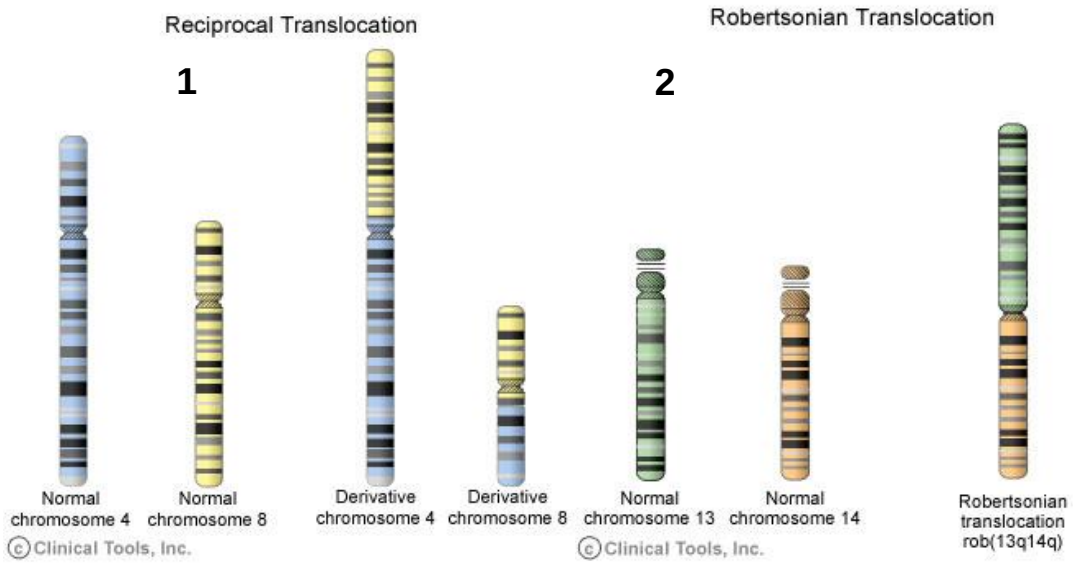

Figure 1: *Chromosomal Translocations*. 1) Example of reciprocal translocation is the rearrangement between two different chromosomes and the regions can be of any size. 2) Example of Robertsonian translocation. It occurs between acrocentric chromosomes (Images taken from Larasig website).

The most common type of chromosomal translocation affecting B-cell cancers is the reciprocal translocation. Usually it involves the region of *IgH*, the immunoglobulin heavy chain region. This gene is very important in the developing of B-cells because it's the origin of the variability of the heavy chain of the antibodies (Robbiani & Nussenzweig, 2013).

To better understand the importance of the chromosomal aberrations study, it's appropriate to spend more words to explain how they occur. The first scientist who

demonstrated the presence of the translocations was Karl Sax in 1938 (Sax, 1938). But it took over 20 years for Peter Nowell *et al.* to consider the relation between the chromosomal aberrations and cancer, leading to the discovery of the association of Philadelphia chromosome with Chronic Myeloid Leukaemia (CML) (Byrne M, 2014). Although the precise molecular mechanisms are not recognized and many processes have been considered, it's now experimentally clear that in order for chromosomal rearrangement to happen, there should be three particular situations: i) double-strand breaks (DSBs), have to occur to create free ends (Aplan, 2006); ii) the free ends have to be next one to each other and iii) the DSBs have to be repaired (Nussenzweig & Nussenzweig, 2010). Sometimes normal chromosomal rearrangements are the cause of the translocations and they occur during the class switch recombination (CSR) or somatic hypermutation (SH). Both of them bring to formation of free ends of DNA that can be repaired by different pathways. One of these is called non-homologous end joining (NHEJ). The complex involved in the pathway is composed by DNA-dependent protein kinase, ligase complex and other factor. All together they ensure the pairing of the two ends of DNA. The complex acts without a template of double strand of DNA (Van Gent & Van Der Burg, 2007).

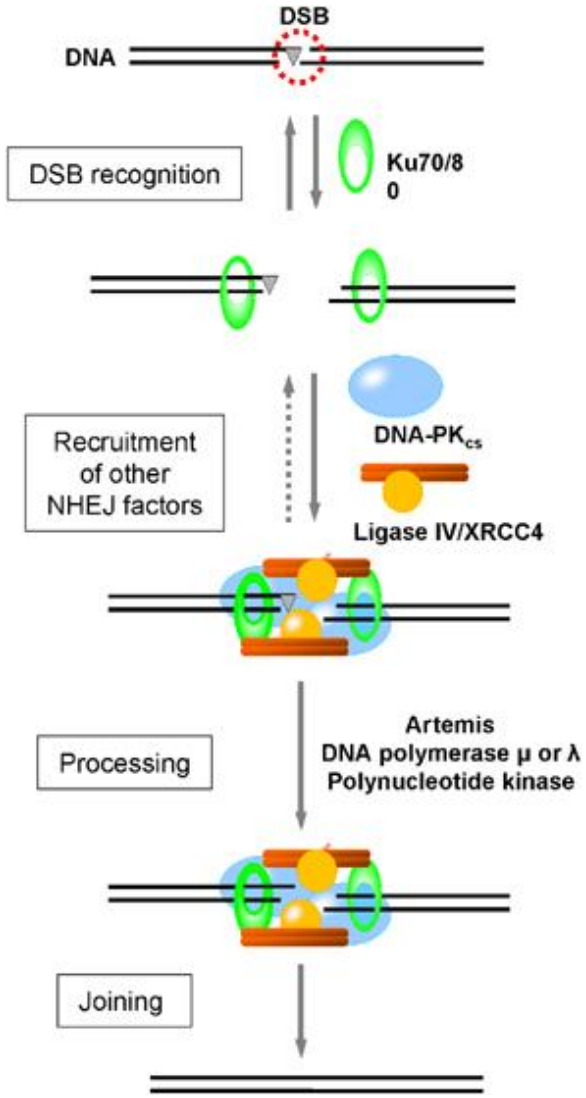

Figure 2: *Pairing of two free end of DNA, non-homologous end joining.* The picture shows the mechanism through which the two free ends of DNA are repaired by non-homologous end joining (NHEJ) (Van Gent & Van Der Burg, 2007).

Although it acts as repairer of DNA, it can happen that one variant of NHEJ is activated and promotes the translocations, specifically aNHEJ (alternative non-homologous end joining) (Van Gent & Van Der Burg, 2007). The process is still not clear, but it's thought to play an important role in the formation of translocations.

The classic NHEJ is also used in B cells to repair two ends broken by enzymes RAG (Recombination-activating genes) during the recombination V(D)J. The first stage of the formation of antibodies occurs during the V(D)J process. Two recombinases RAG 1 and 2 are responsible of assembly and nick of the variable, diversity and joining segments. Following this process, the NHEJ process repairs the two free ends of DNA. Otherwise the maturation of B-cell is affected by the

action of AID (activation-induced cytidine deaminase) enzyme, which is responsible of hypermutation of B-cells in germinal centers. Both these two processes are strictly controlled, but occasionally the loss of some components or elements of the checkpoints can lead to breaks in the DNA without repair (Nussenzweig & Nussenzweig, 2010). It was found that the enzymes are responsible of breakage also in non-Ig genes. For instance, the *BCL2* gene that is involved in t(14;18) translocation is cleaved by RAG enzymes at pre B-cell stage (Nambiar & Raghavan, 2012).

Although processes of formation of chromosomal translocation are still barely elucidated, it's clear that the B cells are easily subjected to rearrangements because of many mutations and nicks from the early stage to the maturation.

Other observations had brought to the evidence that genes closely located inside the cell nucleus have more probability to join and produce translocations. Roix and colleagues conducted an important study in order to see if the spatial separation in the normal cells could be relevant factor in the formation of translocations (Roix, et al., 2003).

1.2 Spatial nuclear arrangement

The formation of chromosomal translocations is influenced also from the proximity of the involved chromosomes inside of the nucleus. The spatial nuclear organization is a well-defined structure, but it is characterised by a non-rigid conformation. This allows that the chromosomes can move inside of the nuclei and therefore the chromosomal translocation can happen also between two chromosomes which normally have different locations (Bickmore, 2013). The chromosomes are located based on different features. The knowledge of non-random localization of chromosomes is one of the most important discoveries of the past years. The organization influence many aspects of the genomic functions (Misteli, 2007). Particularly, it is known that the spatial disposition of translocation-prone chromosomes influences the formation of chromosomal aberrations.

Although the study of nuclear organization developed mainly in the last 20 years, the first observations of nuclear arrangement were made in the 19th century. Rabl and colleagues were the first to discover it, but only around 15 years later Bovari termed Chromosomal Territories (CT) (Figure 3). He told in his studies that every chromosome in the interphase had its individuality and its specific site in the nucleus. In order to see the nuclear organization he used the protrusions of chromosomes in prophase nuclei as markers and he could claim the following observations still valid: I) the CTs are constant in interphase; II) the CTs change during the metaphase; III) the changed pattern of chromosome neighbourhood is then stable in the successive phases of the cell cycle (Cremer & Cremer, 2010).

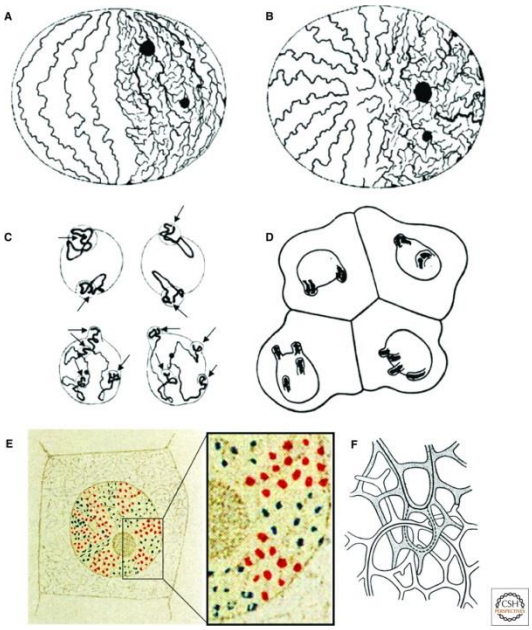

Figure 3: *Drawing of the first chromosome territories*. The first drawings of chromosomal territories by Rabl in 1885 (Cremer & Cremer, 2010).

Only 100 years later, it was possible to visualize directly the chromosome territories inside of the nuclei by DNA FISH (Fluorescent in situ hybridization). During this time, only in the first 80s of the 20th century some scientists started to consider again the idea of Bovari (Cremer, et al., 1982), but they could only experiment that indirectly. With the help of new techniques, began the era of direct observation of single chromosomes in the nucleus (Schardin, et al., 1985). Successively, the improvements in the new techniques allowed to demonstrate the CTs in a three dimensional visualization (Cremer, et al., 2008).

However, the studies about the nuclear organization and how this can affect gene expression are still at the beginning, even if many discoveries have already been made.

One advantage in this discovery was when it was highlighted the radial position of chromosomes (Croft, et al., 1999). It means that the chromosomes are placed along axis from the center to the periphery of the nucleus. The work of Croft and colleagues compared the different positioning of chromosomes 18 and 19 by FISH technique. The two chromosomes are similar, but their localization is different based on their different gene content. The chromosome 18 is a gene-poor one and it's located in the peripheral nucleus, meanwhile the chromosome 19 is gene-rich and it's found in the center. The discovery is founded on a 2D FISH, but later also a 3D FISH confirmed the findings (Cremer & Cremer, 2001). These results agreed also with those where it was found sequences GC-rich close to the central nucleus (Ferreira, et al., 1997). The GC-rich sequences are found in the regions of DNA with major density of genes.

At the beginning, it was also strong the certainty that the chromosome positioning was related to the activation of genes. This one was due to the movement of some genes from the periphery to the center during the activation. The transfer regulated their activity, as it was seen for IgH gene in murine B-cell differentiation (Kosak, et al., 2002). Otherwise, some scientists observed that other genes which even inactivated lacked of the movement to the periphery of the nucleus (Takizawa, et al., 2008). Supporting these findings, it was also the work of Ragoczy and colleagues (Ragoczy, et al., 2006) where they demonstrated the lack of movement of β-globin in the early stage of the activation. Additionally, the heterochromatin was observed also in different locations inside of the nucleus and not only on the edge. The heterochromatin is known to be transcriptionally silent, but some studies showed its positioning to more central positions (Takizawa, et al., 2008) (Figure 4).

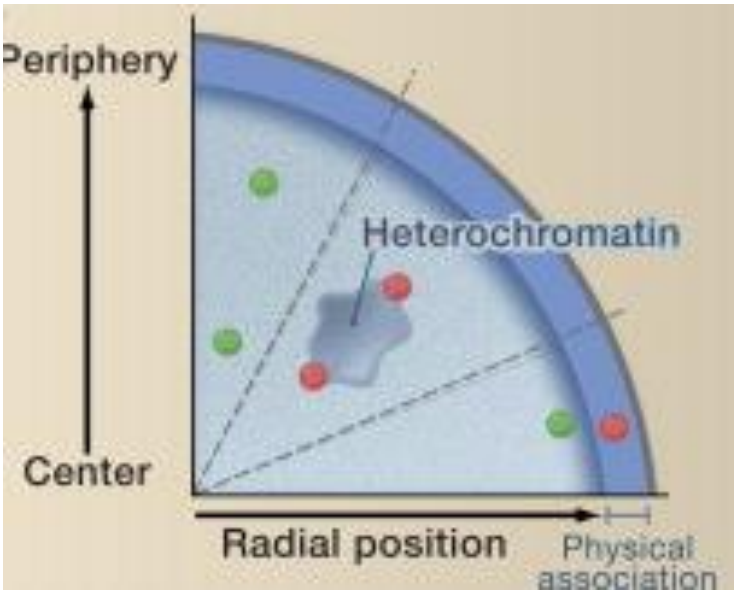

Figure 4*: Radial positioning of chromosomes*. The image shows firstly the position of active genes (green) in different areas of the nucleus; secondly, inactive genes associated to the heterochromatin are in a more central position compared to that expected one. Thirdly, the active genes not physically associated to the periphery can maintain their activity (Takizawa, et al., 2008).

To demonstrate the complete lack of relationship between gene activity and their nuclear location, one approach was made by Kumaran and Spector (Kumaran & Spector, 2008), where they relocated a reporter gene close to the nuclear lamina. After stimulation it was found that its competence of transcription wasn't affected by relocation. One of the better explanations of the active or inactive genes positions at the nuclear envelop is the differences between positioning and association to the periphery of the nucleus. It's known that the major component of nuclear membrane is lamina. The protein is linked to the proteins of heterochromatin which are responsible of the regulation of the heterochromatin (Takizawa, et al., 2008). Consequently, it was demonstrated that the association of chromosomes to the lamina leads to loss of activity of the genes.

In the 1985 Blobel and colleagues hypothesized the "gene-gating" hypothesis (Kalverda, et al., 2008). He enunciated that the three dimensional structure of the chromatin was due to the nuclear envelop. Specifically, the detection of non-random distribution of nuclear pore complex (NPC) along with peripheral nuclear lamina and other components of the nuclear core could probably show the cause of the nuclear arrangement. He assumed that transcriptional units of activated

genes were linked to a nucleoporins. Consequently, the gene-gating activity of nuclear pore complex allows the transcripts to exit in the cytoplasm. (Blobel, 1985). Obviously, the techniques in use on that time couldn't demonstrate Blobel's theory, but later new studies confirmed it in part.

However, it was demonstrated that the radial positions of genes is not always stable. By the DamID (DNA adenine methyltransferase identification) technique, which takes the bacterial DNA adenine metiltransferase linked to membrane protein, it was possible to see that the radial position was cell-specific and sometimes the gene is released by the nuclear peripheral proteins for following activation (Peric-Hupkes, et al., 2010). The technique allowed building a high-resolution map of the interaction between chromosomes and nuclear lamin in murine stem cells. The method tried to clarify the difficult visualization of this interaction and its dynamics in the nucleus.

Additionally, some studies revealed that the movement or lack of movement of some genes depends on their function. Those involved in differentiation are more prone to relocalization based on their expression, for example IgH (Kosak, et al., 2002). On the other hand, the movement of some genes doesn't change based on their expression, for example Bcl-2 gene (Meaburn & Misteli, 2008). The genes of this group are not completely silenced during differentiation. Thissuggests that the genes which go under repositioning during activation are those lying on heterochromatin blocks (Takizawa, et al., 2008).

In conclusion, the nuclear structure is likely influenced by the nuclear envelop, that might be the only responsible of three-dimensional organization of chromatin. The presence of nuclear lamin allows the chromatin to be silenced thanks to association of chromatin/lamin. Its role in silencing is almost undiscussed thanks to works as that one from Reddy KL and colleagues (Reddy, et al., 2008) when it was demonstrated by reporter gene that the association of this latter with lamin leads to its silencing as well as the close genes. Additionally, other studies demonstrate that some genes can move in the three-dimensional nuclear structure and this can lead some activated genes to the periphery . Some components of nucleoporin complexes and nuclear envelop could work as insulator between active and inactive chromatin regions and they could contribute to the dynamic state of chromatin (Kalverda, et al., 2008) (Figure 5).

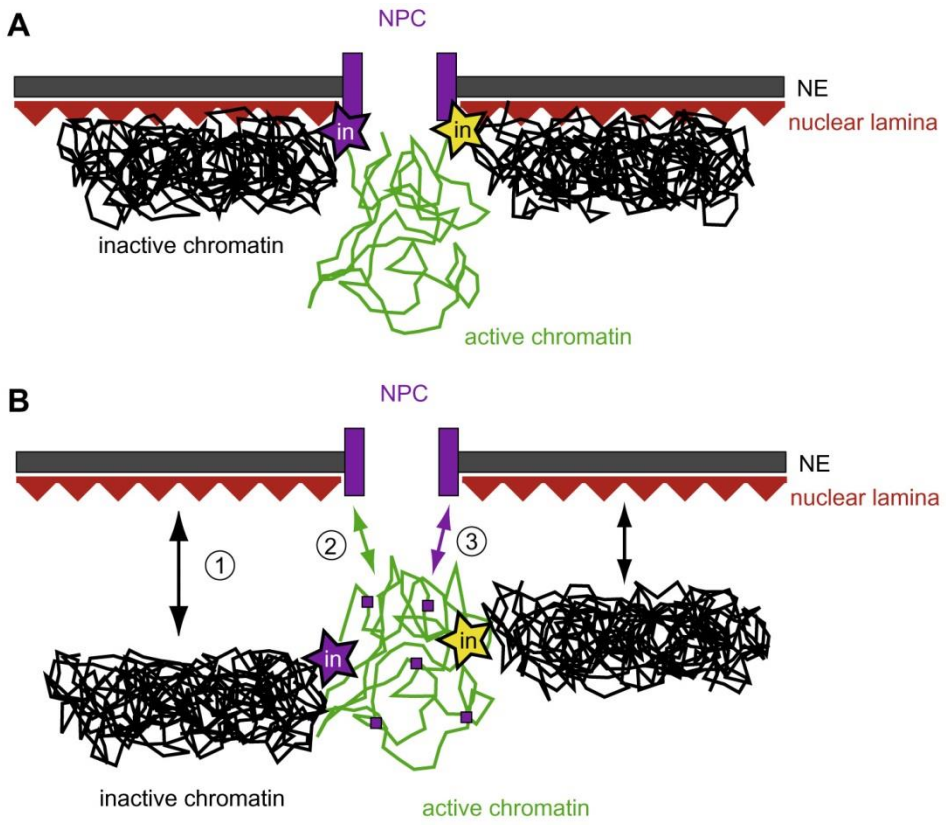

Figure 5: *The dynamic state of chromatin at the nuclear periphery*. A) it's the static chromatin at the nuclear periphery. Inactive state of chromatin is repressed at periphery; some components of NPC could act as insulator between the inactive and active chromatin. B) (1) The dynamic chromatin interacts with nuclear lamina and NPC (2), probably due to different states of nucleosome. Also the components of NPC could help the movement of chromatin (3) (Kalverda, et al., 2008).

1.3 Proximity of translocation-prone gene loci

It is known that the nuclear arrangement influences the event of chromosomal translocation.. Many studies are developing in this direction to figure out whether neighborhood patterns are responsible for the formation of aberrations. As mentioned, the chromosomes tend to occupy specific regions inside the nucleus. These regions are defined as chromosome territories and are localized non-randomly. The position of every territory depends on the density of genes in the chromosome. If a chromosome is gene-poor is found preferentially at the

periphery of the nucleus; the contrary for the gene-rich chromosomes (Croft, et al., 1999). Also the gene positioning follows specific patterns and they are cell and tissue specific, in other words it depends for example on the degree of differentiation of the cell (Marshall, 2003).

The nuclear organization is non-random and this led the scientists to try to understand if the neighbourhood of the chromosomes was preferential. The rationale led scientists to analyse if it was possible to detect a relationship between the spatial arrangement and preferential rearrangements between two different chromosomes. In 2002 Parada and colleagues (Parada, et al., 2002) studied if in normal and tumoral cells the proximity of two chromosomes could influence the formation of translocations. They hypothesized that if the chromosomes lay in preferential locations in the nucleus, closer chromosomes together should have a higher chance to form reciprocal translocations. The theory was also sustained by the detection of close genes involved in a translocation before the event. They studied a lymphoma cell line where they saw that two translocated genes were preferentially located close one to each other. The same evidence was observed in normal splenocytes. Using two different methods to see the preferential proximity of three chromosomes involved in two translocations, the authors demonstrated that in normal and tumoral cells the spatial chromosome positioning was preferential, but not in every case. They claimed that the proximity could be dependent on the tissue analysed and could be also cell specific (Parada, et al., 2002). They claimed that their studies supported the theory that the proximal localization of chromosomes makes easier the chromosomal rearrangements. Supportive of this, another previous study on RET and H4 genes involved in a chromosomal translocation in papillary thyroid cancer was seen the proximity of the two chromosomes only in the specific tissue, but not in mammary cells where the rearrangement doesn’t occur (Nikiforova, et al., 2000).

The higher-order of chromatin is also due to the association with proteins along fibres of chromatin. Some of the proteins are responsible for the looping out of the chromosome fibres leading to distinct genomic regions next to others. It was shown that these chromosome regions are more affected by DSBs and this is probably why they are more subjected to translocations (Misteli, 2010). One of the

reasons leading to the cleavage of the DNA sequences looped out is the decondensed nature of the chromatin loops and additionally the intermingling area was seen in correlation with translocation (Branco & Pombo, 2006). By a high-resolution in situ hybridization procedure they showed that different chromosomal territories are often mixed together. The frequency of intermingling is correlated to the frequency of chromosomal translocations in the same cell type. It was assumed that this finding along with the presence of many transcription factories in the intermingling areas could affect the genome stability in specific cell types.

In line with this finding, some studies analysed the movement of DSBs in cancer cell lines. The study was performed to understand if the DNA joining after breakage could occur at long distances or if a proximal neighbour chromosome was preferred (Soutoglou, et al., 2007). These experiments showed that the DSBs do not move more than 250 nm before finding another free end for the translocation. The stable position of DSBs can be seen as protection. If the free ends could move everywhere in the nucleus, the consequence would be frequent rearrangements every time there is a cleavage (Soutoglou & Misteli, 2007) (Figure 6).

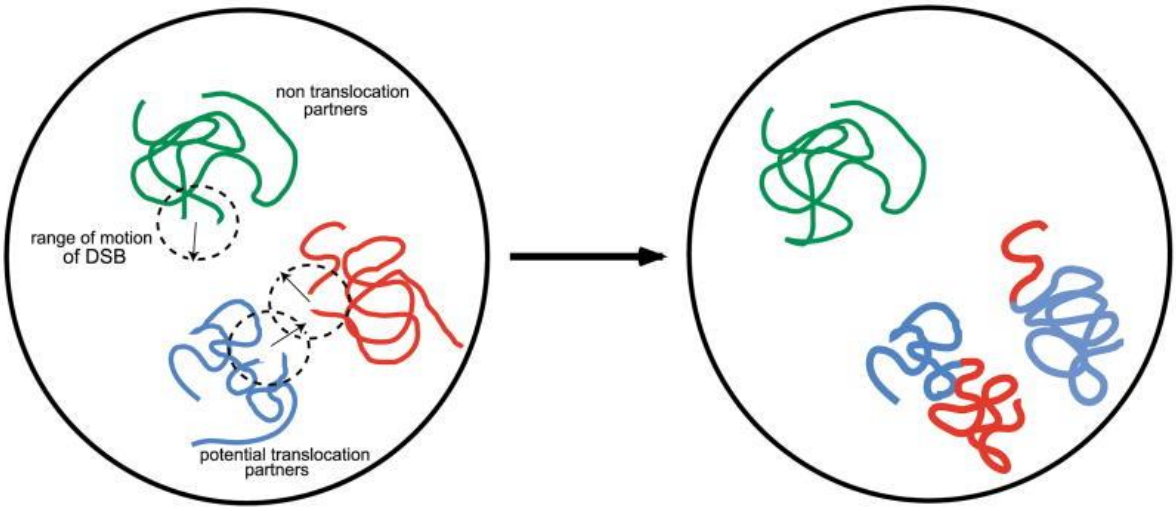

Figure 6: *The spatial immobility in the formation of translocation*. The distant chromosomes cannot form chromosomal translocations because of the immobility of DSBs in the long distance (Soutoglou & Misteli, 2007).

The work is based on the two preferential models of formation of translocation (Figure 7). The first model is named “contact-first”; this is based on a static model according to which the free ends don’t go too far from the breakage before being

sealed with other free ends. It means that the translocations require a pre-existing proximity of the two chromosomes involved in the nucleus. This theory looks like more supported in mammalian cells where the higher frequency of translocation is between two close chromosomes. The second theory is based on a dynamic connection of two distinct free ends of DNA. The model is named “breakage –first” and it says that the genome regions undergo firstly to breakage and then they move around in the nucleus to find another free end. This theory was shown in a yeast study, where the motion of free ends of DNA was supported (Lisby, et al., 2003), but it doesn’t look like valid in the mammalian cells.

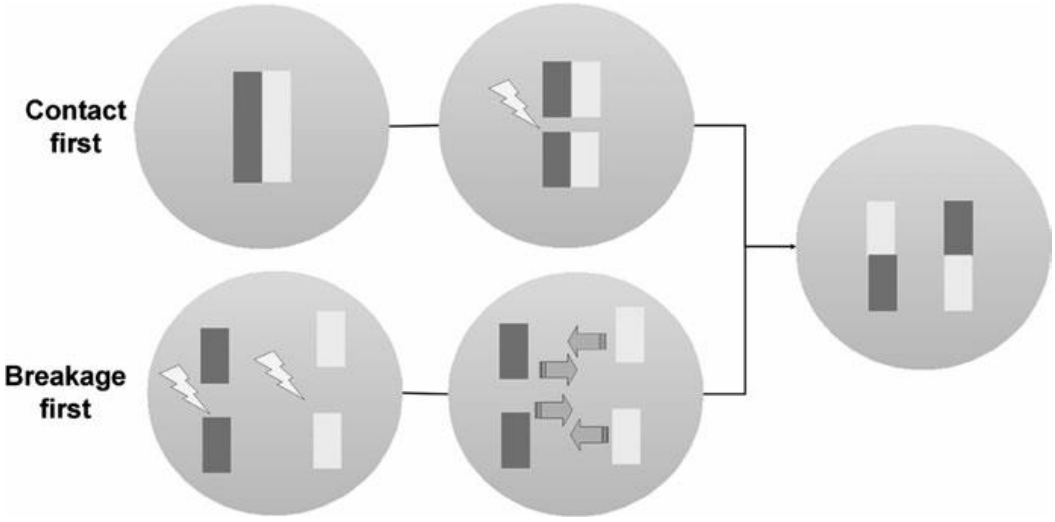

Figure 7: *The two model of formation of translocations*. On the first row, it’s showed the first model of formation of chromosomal translocation. It’s named “contact-first” and it’s based on a static theory of meeting of free ends on two different chromsomes. On the second line, it’s illustrated the second model, “breakage-first”, that it’s based on a dynamic view for linking the two free ends involved in a translocation (Soutoglou & Misteli, 2008).

The importance of the spatial arrangement for the formation of chromosomal translocation was also shown in pre-B cells to demonstrate which are the preferential breakpoints and joining on chromosomes. Mahowald and colleagues studied if the frequency of aberrantly joining in $ATM^{-/-}$ mice was due to RAG breaks. The study was made by a system to minimize the selection biases leading to a huge amount of breakpoints. They claimed that the aberrations and their joining occurred frequently because of RAGs activity on specific chromosomal regions. Moreover, RAGs aberrantly activity in pre-B cells on the same

chromosome leads to small deletions rather than translocations.(Mahowald, et al., 2009).

Another example for the relationships of spatial proximity and translocations in B cells was originated by the work of Roix and colleagues in 2003. They observed that the c-myc translocated often to the *IGH* gene and this was due to the proximity of the two genes. Otherwise, c-myc gene moved rarely to the IgK gene because of the distance in the nucleus (Roix, et al., 2003). In the same work, they observed similar results for other translocations and the finding could step up the importance of the genome proximity. Other works supporting this theory are related to the *BCR-ABL* translocation. It's known that this is typical rearrangement of chronic myelogenous leukaemia and it happens in the early hematopoietic stem cells. In a study of 1999, Neves et al. observed the distance and the frequency of translocations between *BCR* and *ABL* genes and *PML* and *RARα* genes, typical aberration of acute promyelocytic leukaemia. They observed the cells in different stages of differentiation and of cell cycle using by hybridization in situ and confocal microscopy. They found that in the early hematopoietic stem cells *BCR* and *ABL* genes are close, but not the other pair of genes (Neves, et al., 1999). They were able to figure out the importance of proximity of the genes involved in translocation and they related this finding to the stages of the differentiation of the cells.

The aberrations and the proximity of the involved genes don't look like dependent only from the type of the cells and stage of differentiation. A new study of the fusion of *TMPRSS2-ERG* declared that the spatial proximity can be induced also by signalling. The *TMPRSS2-ERG* is a typical translocation of prostate cancer and it involves a member of ETS family genes and androgen transmembrane receptor. The scientists treated the prostate cancer cell lines with dihydrotestosterone (DHT) and they observed that by myosin and actin-dependent mechanism the two genes get closer one to each other. Moreover, after irradiation-induced breakages the cells presented high frequency of the translocation (Mani, et al., 2009).

All these findings highlight that the higher-order of chromatin in the nuclei plays an important role in the formation of chromosomal translocation. However, it's still unclear how all these mechanisms can happen. The rearrangements, indeed, can be produced by several factors due to external and internal mechanisms and environment and to a combination of those (Figure 8).

Figure 8: *Factors triggering chromosomal rearrangements*. The ring conceptualizes the several factors influencing the production of chromosomal translocations. In the inner there are the principal categories; in the outer there are specified the specific factors for every category (Mani & Chinnaiyan, 2010).

1.4 t(14;18) translocation

The study of chromosomal translocations is one of the most promising ways for studying the molecular mechanism leading to the insurgence of cancer. This approach made it possible to discover new cancer genes, among which it is *BCL2*. The gene was firstly found at the translocation breakpoint in a newly identified translocation t(14;18) with *IgH* gene, immunoglobulin heavy chain region joining (Tsujimoto, et al., 1985). The rearrangement involves the *BCL2* gene on chromosome 18, particularly 18q21 region, and *IgH* gene on chromosome 14q32 (Figure 9). The *BCL2* (B-Cell CLL/Lymphoma 2) codifies for an integral protein of mitochondrial membrane and it is part of the *Bcl-2* family responsible for regulation of apoptosis. Specifically *BCL2* is anti-apoptotic protein and regulates the cell death through the permeability of mitochondrial membrane. The fusion gene was found in follicular lymphoma cells and diffuse large B cells lymphoma (DLBCL).

The consequence of the translocation of *BCL2* in proximity of IgH promoter is a constitutive activation of *BCL2* in the cells.

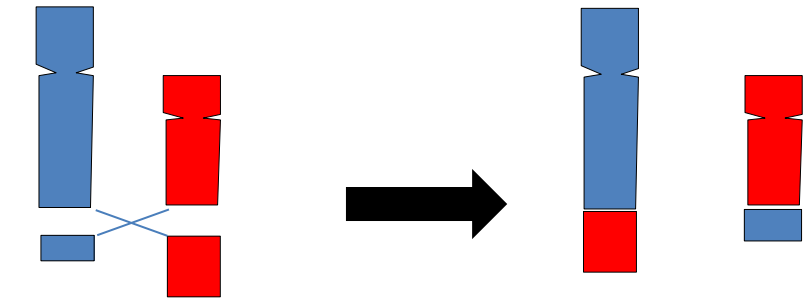

Figure 9: *chromosomal translocation t(14;18) (14q32;18q21)*. The image summarizes the translocation leading to the formation of der(14) and der(18). The der(14) is responsible for the activation of Bcl2.

The breakages on chromosome 14 and 18 bring to the formation of two derivatives: der(14) and der(18). The first one is characterized by the transfer of *BCL2* close to the *IgH* gene. The result of the translocation is an overexpression of bcl2 protein regulating the cell death in proliferating and differentiating cells. It is expressed amply in fetal tissue, but limitedly in the adult organism. *BCL2* is highly found in pro-B cells and mature B-cells, but its expression decreases in the B cells in maturation and those preparing to expose the immunoglobulin on the surface (Merino, et al., 1994).

BCL2 is a member of the BCL2 family. The components of whole group are apoptosis regulators and they can divide in repressors as BCL2 and activators, as BAX and BAK. In particular, BCL2 codifies a protein that it leads to the inhibition of the apoptosis. It acts preserving the integrity of mitochondrial membrane; indeed, it blocks the oligomerization of BAX/BAK that causes the release of apoptotic factors from mitochondria on the outer membrane. Additionally, it binds inner stress sensor BIM and Puma that activate in turn BAX and BAK to promote the apoptosis (Correia, et al., 2015). Since the *IGH* gene is activated in B cells, the translocation induces a constitutive activation of the *BCL2* locus and consequently to an overexpression of the anti-apoptotic protein (Kridel & al., 2012). Transgenic mice

that overexpress *bcl2* show a high reduction of apoptosis in B cells as the wild type mice do (Smith & al, 2000). This means that the constitutive activation of *BCL2* alters the GC (germinal center) and memory B cells dynamics (Swaminathan & Müschen, 2014). Obviously, the alteration of *BCL2* cannot arise alone the follicular lymphomas, but cooperating lesions help to develop the disease. An example is given by AID activity, which was seen as relevant factor along with the presence of t(14;18) (Hirt, et al., 2007).

It's important to explain deeply how the translocation of *IGH/BCL2* occurs at molecular level. The breakage of chromosome 14 is in the Joining region genes (J_H) of the IgH locus. The breakage appears during the V(D)J recombination that it brings to the variety of immunoglobulins set. One of the responsible of the recombination is AID, activation-induced deaminase, which is expressed in B cells. It is not only the responsible of the mutations and variation of the receptors of B cells, but it also acts mutating genes outside the immunoglobulin locus as for example *BCL6* and *MYC*. Also these genes were found associated to chromosomal translocations along with immunoglobulin locus (Halldòrsdòttir, et al., 2008).

To better understand the strong relationship between the *IGH/BCL2* translocation and AID, Sungalee and colleagues developed a murine model to study the genesis of follicular lymphomas (Sungalee, et al., 2014). They analysed that the memory B cells can reenter and reengage the GC B cells (germinal center B cells). This dynamic can lead to an increased selection of cells with translocations, considering that they have a bigger chance to survive compared to the cells without an overexpression of *BCL2* (Figure 10).

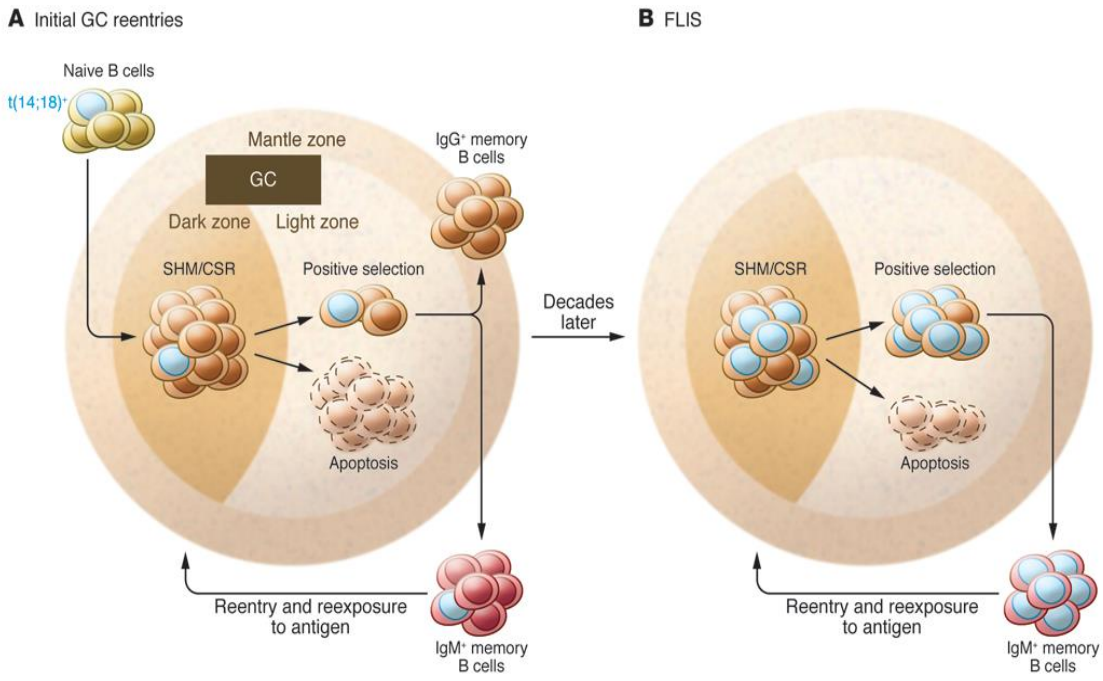

Figure 10: *The memory B cells reenter in GC and increase the selection of t(14;18) cells*. A) Normal and t(14;18) cells enter in GC reactions and by SHM and CSR there is the first positive selection. The naïve B cells are more led to be selected positively thanks to the overexpression of Bcl2 causing lack of apoptosis. B) After decade selections, the naïve B cells will be more compared to the normal cells and this causes the onset of Follicular Lymphomas (Swaminathan & Müschen, 2014).

The memory B cells reenter several times to GC dynamics and the overexpression of *BCL2* favours the selection of the naïve B cells that they have the chromosomal translocation *IGH/BCL2*. At the end, the scientists saw that the repeated GC transit along with the activity of AID bring to the onset of follicular lymphomas (Swaminathan & Müschen, 2014).

1.5 FISH techniques through history

The technique used mostly to detect DNA and RNA in the nuclei is Fluorescent in situ hybridization (FISH). Through the decades, this technique has been developed and new efforts are used for a more accurate detection of the target. The method (Figure 11) consists in the use of probes able to recognize and bind specific targets. The detection occurs by fluorescent dye bound to the probes and

the researchers can see and analyse the results to specific fluorescent microscopes with selected filters.

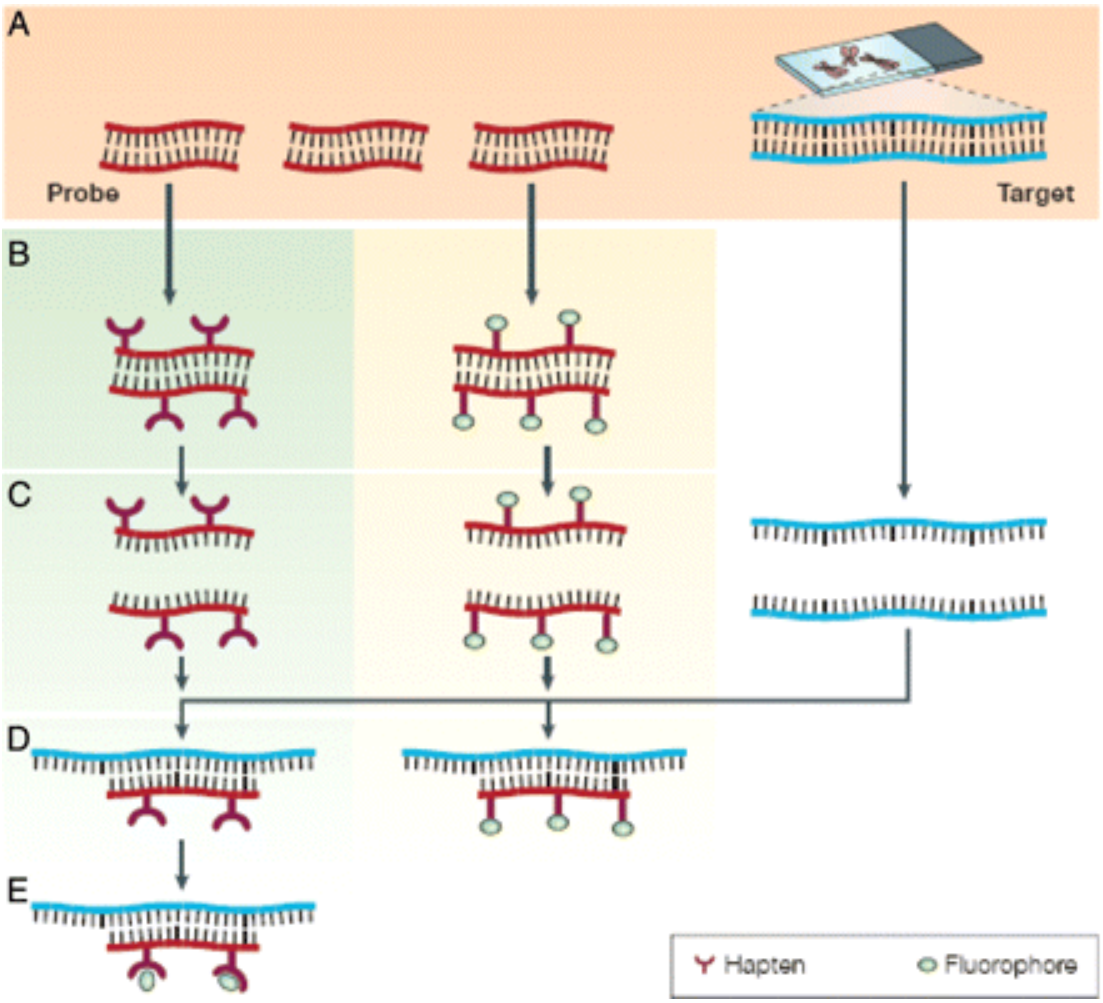

Figure 11: *Fluorescent in situ hybridization (FISH) method scheme.* The image shows the schematic representation of Fluorescent in situ hybridization (FISH). The technique starts with the fixation of cells on slides; then, the cells and the probes are denaturated to be hybridized. The probes are previously labelled by hapten and then they are detected by binding to fluorophores. They can be also directly labelled by specific fluorophores. The hybridization occurs by an incubation of cells and probes together at 37°C in humidified chamber. After the hybridization, there is the step of detection by fluorescent molecules. If the probes are directly labelled the step is skipped. Then, the slides are washed to eliminate the not hybridized probes. At the end, the target is detected at specific fluorescence microscope set by filters to visualize the probes (Bishop, 2010).

The first application of hybridization to a DNA target happened in the last 70s where an RNA probe was hybridised to a DNA target (Gall & Pardue, 1969) (Rudkin & Stollar, 1977). The first improvement was made by Langer and

colleagues in 1981 where for the first time they used an amino-allyl modified base allowing reducing the noise and detected by hapten or fluorophore conjugated. Further efforts were made always in 1980s when the indirect labelling brought to an increase of the level of the signal (Singer & Ward, 1982), but only ten years later it was possible to create synthetic probes for a better selection of the specific target (Kislauskis, et al., 1993). The first probes were really large due to the methods used to obtain and this could lead to a high signal from background. The later approach to clean the nucleotide not conjugated allowed to overcome the issue and to define the whole chromosome painting (Lichter, et al., 1988). The “chromosome painting” was one of the first applications of the FISH technique (Pinkel, et al., 1988). It consists in using as probes entire libraries for chromosomes in order to paint different chromosome segments. It is used to visualize chromosome aberration.

A more defined probes model was developed between the end of the 80s and 1990s. At that time many efforts allowed to reduce the size of the probes able to improve the signal-to-noise ratio and the specificity of the detection. Moreover, these improvements had been applied to the detection of the RNA (Femino, et al., 1998). From the same years the new approaches as multiplex fluorescence in situ hybridization were experimented in order to detect more targets together. The technique is called M-FISH and it is 24-colours procedure utilized principally in karyotyping (Nederlof, et al., 1990). Summarizing, there are many different types of probes and above all they differ by size. The choice of the probes is really important before starting an analysis by FISH. The probes are essentially divided in three categories: i) the locus-specific probes, binding a specific region of chromosome; ii) repetitive sequences probes, based on repetitive sequences found in the middle of chromosomes or at the end of chromosomes, or in sequences as Alu and LINEs; and iii) whole chromosome probes, group of smaller probes hybridizing a whole chromosome together (Bishop, 2010) (Table 1). The different probes are also used in combination to visualize different DNA target.

Types of probes	Applications	Origin
Locus-specific	Structural aberrations of metaphase and	Cloning vectors, as plasmids, BACs (bacterial

	interphase chromosomes	artificial chromosomes), PAC (P1-derived artificial chromosomes)
Repetitive sequences	Visualizing telomeric and centromeric regions of chromosomes	Pericentromeric bacterial artificial chromosomes (BAC), plasmids
Whole chromosome	Chromosome aberrations in metaphase, M-FISH (multicolour-FISH) and SKY (spectral karyotyping)	Derive by single chromosome amplified and labelled by PCR

Table 1: *Different types of FISH probes*. The table illustrates the different types of FISH probes, along with their applications and origin of production.

The fast improvement of FISH and continuous research of more targets in the same experiments were not only referred to DNA, but they were started to use also for following the nascent transcripts. In the 2002, Levsky and colleagues studied the gene expression by a combination of multi-colours together (Levsky & Singer, 2003).

The RNA FISH is a technique more and more developed and refined to visualize the gene expression in quantitative and qualitative way. Above all, by RNA FISH it has been possible to see the right localization of transcripts inside the cells. Initially, it was tough to visualize directly the RNAs because of the low abundance of some of them inside of single cells. In order to develop the accuracy, the researchers developed many new approaches for visualizing single molecules of RNA. One of them was to use more than one short oligonucleotide complementary to a short region of the target. Every single probe was labelled with the same fluorophore and every probe was complementary to different regions (Raj, et al., 2008). The technique is useful to detect for example RNA molecules as mRNA molecules in fixed and permeabilized cells. New efforts tried to see the signals from transcripts trying to circumvent the issue of the low signal. Further improvements to increase the strength of the signal were also to use the combination of PCR technique with Fluorescence in situ hybridization. It's defined

“in situ PCR” and even if it’s not a tight FISH technique could be really useful for increase the power of RNA signals. Unfortunately, there are many problems to allow the Taq polymerase can arrive in the right site for the amplification (Zwirglmaier, 2005). A good improvement has been to use direct labelled probes to detect the new transcripts inside of the single cells. The new effort allowed seeing a stronger signal, trying to reduce the signal-to noise ratio.

The detection of transcripts by FISH technique has been coupled to detection of their DNA target. The combination of two techniques allowed seeing the importance of spatial nuclear arrangement of some genes and understanding which nuclear position is preferential in order to be transcribed. The combined RNA/DNA FISH was used for example in the study of murine β-globulin locus during the erythroid cells maturation (Ragoczy, et al., 2006). Thanks to this technique, scientists were able to prove that the nuclear periphery is transcriptionally active contrarily to previous findings.

Also Chaumeil and colleagues used the RNA-DNA FISH associated to immunofluorescence (immune_FISH) (Chaumeil, et al., 2008). In their work on X-chromosome inactivation, good model for representing the investigation of the formation of facultative heterochromatin, they used immuno- FISH in order to understand the kinetics of the events leading to X inactivation. Although the difficulties met to combine three different techniques and allow the probes to enter in the nuclei, the FISH techniques are clearly the most reliable methods to see directly the gene expression along with own transcriptionally active gene.

1.6 Background of the project

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Previous studies leading to this project addressed the question whether, as a consequence of chromosomal translocations, there is an alteration in the nuclear positioning of the genomic regions involved in the rearrangement compared to the non-translocated alleles. One of these studies, focused on the relative positions of the genes involved in the chromosomal translocation t(14;18) and their relative

chromosome territories using Fluorescence in situ hybridization (Garimberti & al, 2009).

Two dimensional (2D) DNA FISH using whole chromosome painting probes (Figure 12) was carried out to visualise chromosome territories of chromosomes 14 and 18 in normal peripheral blood lymphocytes from a number of healthy volunteers and in the Pfeiffer cell line.

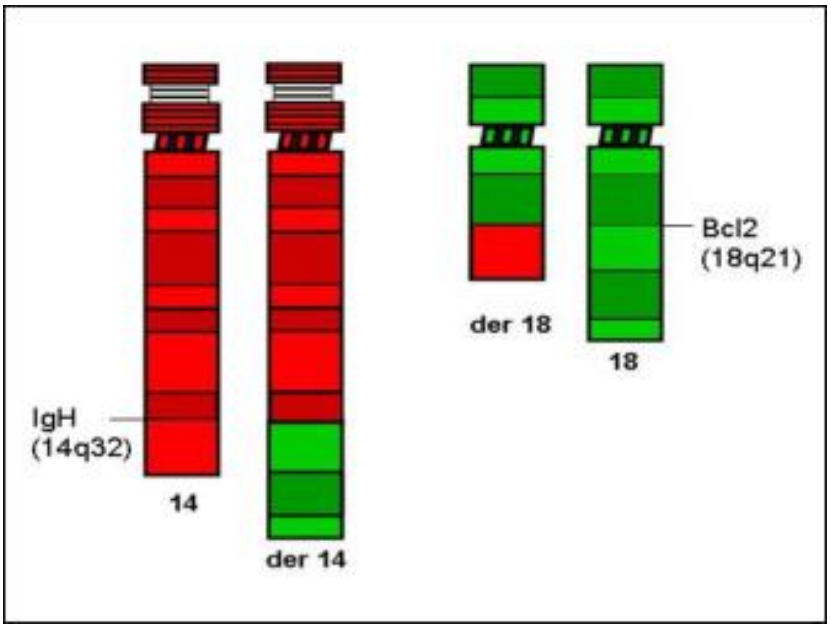


Figure 12: *Whole chromosome painting probes for 14 and 18.* The whole chromosome painting probes are used by Elisa G. for detecting the chromosome territories of 14, 18 and der(14) and der(18) inside of Pfeiffer and control cell lines (Images and data taken from (Garimberti & al, 2009)).

The Pfeiffer cell line was established in 1992 from a patient in the leukemic phase of diffuse large cell lymphoma (DLCL) and express the *BCL2* gene due to the presence of the t(14;18). The study conducted by Garimberti showed no difference in the nuclear localizations of neither chromosome 14 nor 18 territories between controls and both of the cell lines.

Further analysis was been led ahead to detect the genes involved in this translocation, *BCL2* and *IGH* (Figure 13). In the control cells, both of those genes were localised inside of their own territories.

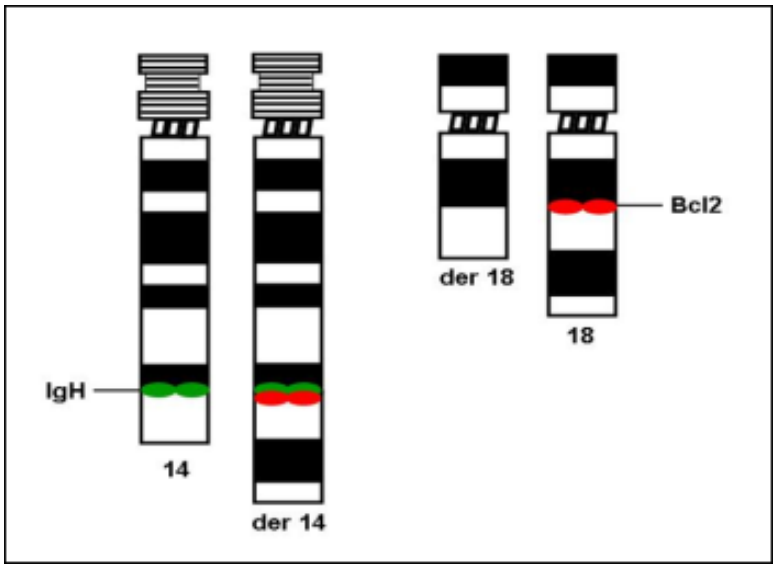


Figure 13: *998D24 and RP11-299P2 probes, for detecting IgH and Bcl2 respectively*. The probes used by Elisa Garimberti for detecting the gene *IGH, BCL2* and the fusion gene in own chromosomal territories in Pfeiffer and control cell lines (Images and data taken from (Garimberti & al, 2009)).

In the Pfeiffer cell line, two findings were of relevant interest. First of all, the translocated *BCL2* gene was found in two different domains and consequently this brought to bimodal distribution (Figure 14). In some populations of Pfeiffer cells the translocated *BCL2* gene was found in the nuclear periphery, where normally the *BCL2* gene is, and in others the translocated *BCL2* gene was found in the central part of the nuclei, where *IgH* normally is. This was probably a reflection of different gene expression in the two different populations.

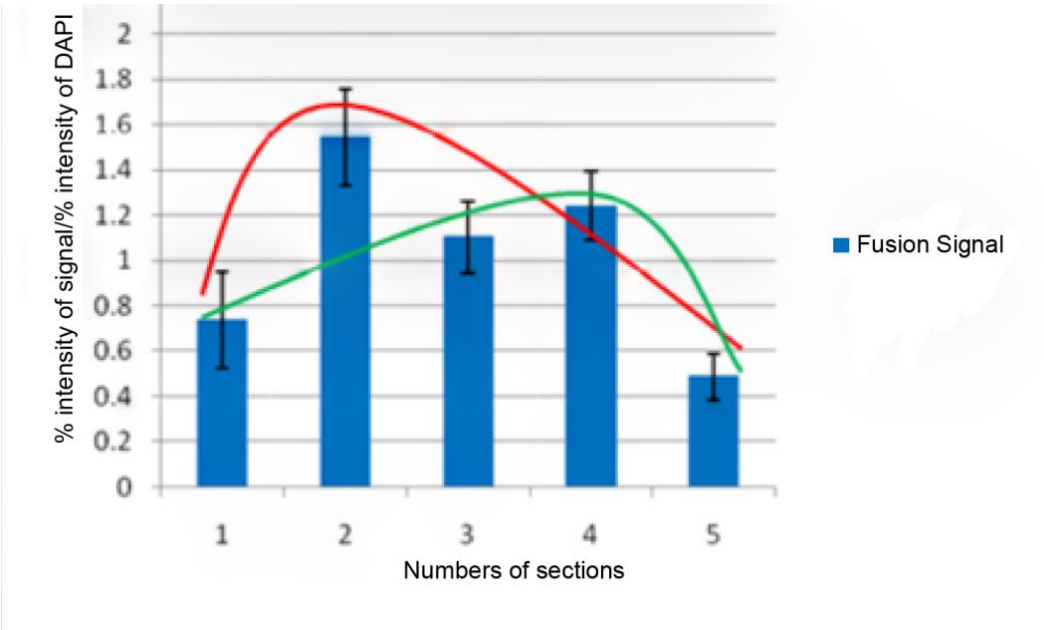


Figure 14: *Bimodal distribution of the translocated gene in Pfeiffer cell line*. The ideogram shows the bimodal distribution of the translocated gene: in some of the nuclei its position is more peripheral, where there usually are present the gene less transcriptionally active; in other nuclei is more central, where the more transcriptionally active genes are (Images and data taken from (Garimberti & al, 2009)).

Further to this analysis, both genes *BCL2* and *IgH* and the tranlsocated alleles were observed in relation to their own territories. The FISH experiments were performed using the probe for *IgH* along with whole chromosome paint probe for chromosome 14 and the probe for *BCL2* along with whole chromosome paint of chromosome 18 (Figure 15). This latter experiment highlighted a further result: in the 15% of nuclei of Pfeiffer the gene *BCL2* was out of its territory. The hypothesis deriving from this work was that the looping out of the *BCL2* gene from its territory could be the reason of the more transcriptionally active gene in some populations of Pfeiffer. In the following table the result of the detection of *BCL2* gene localization related to its chromosome territory is illustrated (Figure 15). The total pictures taken are 71: 0% of the nuclei shows both of the alleles out of chromosome 18 territory, 84.5% of the nuclei has both of the alleles inside of its chromosome territory and 15% of nuclei has one internal signal and the other one of *BCL2* is external to its chromosome territory.

Total pictures taken	Chromosome territory of control		Derivative 14	
	internal signal	external signal	internal signal	external signal
71	71 (100%)	0 (0%)	60 (84.5%)	11 (15%)

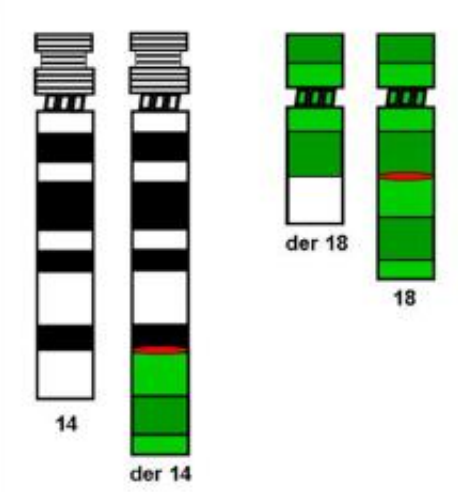


Figure 15: *Above: data of the finding for detection the BCL2 in own chromosome territory; below: image of the probes used.* The table shows the percentage of nuclei showing *BCL2* signals internal or external to chromosome 18 territory in normal controls and in the Pfeiffer cell line. The image shows in red the probes specific for the *BCL2* gene and the chromosome 18 territory respectively. (Data produced by (Garimberti & al, 2009).

Chapter 2

PROJECT AIM

2.1. Aim to the project

The aim of this project is to find the origin of Bcl2 nascent transcripts inside the Pfeiffer nuclei. The cell line has t(14;18) translocation typical of follicular lymphoma that carries out an overexpression of the anti-apoptotic protein *BCL2*, due to juxtaposition of transcriptional unit of *BCL2* to promotor region of *IGH*.

The findings from previous study (Garimberti & al, 2009) highlighted that in the 15% of cases the *BCL2* gene loops out from its territory and this one matches with translocation. The hypothesis was that the translocated *BCL2* was the responsible of the overexpression of *BCL2* and this one was found in a central nuclear position due to its activity. Consequently, we would like to try to set up a technique allowing to visualize the nascent transcripts along with the transcriptionally active genes to understand if the hypothesis from the previous study is correct.

According with the previous findings, we would like to demonstrate that the most of the transcripts are borne from translocated gene. In addition, we would like seeking to determine whether the position of the t(14;18) originating from a looping out of *BCL2*is the preferentially located to a central position of the nucleus.

In order to detect the transcripts and the genes of *BCL2* and *IGH* and the t(14;18), it was decided to set up RNA DNA FISH experiments for a detection in a three dimensional fashion. RNA DNA FISH would enable us to detect the transcripts and the genes simultaneously, by choosing to label each probe a different fluorochrome or dye.

Chapter 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Cell line

The cell line used in this project is CRL2632 Pfeiffer. It's a cell line established in 1992 from a patient affected by DLCL, diffuse large cell lymphoma. The cell line was extracted from metastatic tissue and it's a lymphoblast B lymphocyte cell type. Pfeiffer expresses several chromosomal aberrations, among of which there is t(14;18)(q32;q21) translocation typical of follicular lymphomas. The aberration involves the *IGH* gene (Immunoglobulin heavy-chain) and *BCL2* (B-cell leukaemia-lymphoma). In a study done by Bakhshi et al. (Bakhshi, et al., 1987), it was found a new transcriptional unit on the derivative chromosome 14 that it was recognised as BCL2 gene. The chromosomal juncture juxtaposes the promotor of immunoglobin heavy chain joining region (J_h) to the gene *BCL2*, expressing one of the anti-apoptotic protein (Figure 16), and causing the pathology.

The following image shows the mechanism leading to t(14;18). The proposed scheme is referred to the first study of the said translocation.

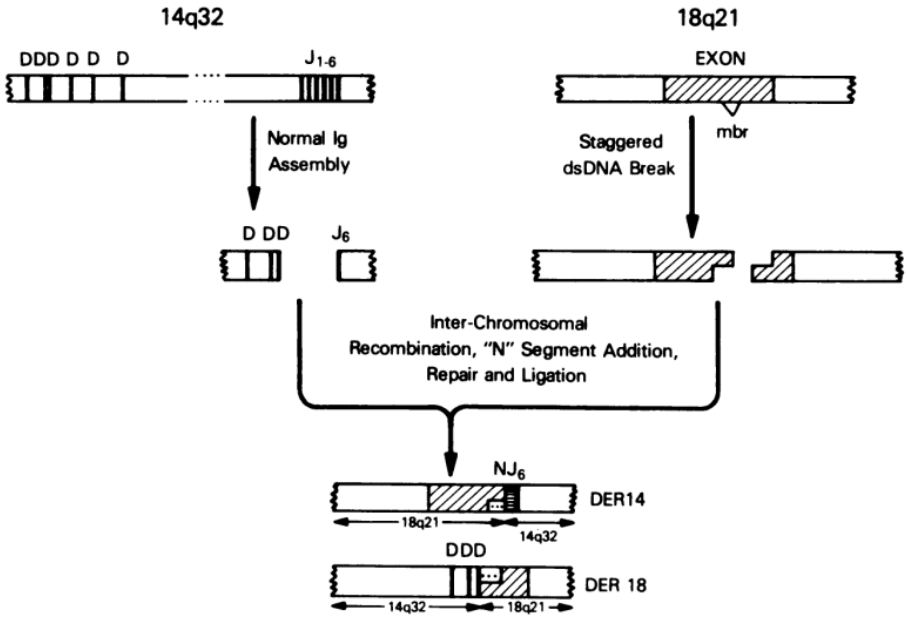


Figure 16: *Mechanism of t(14;18) translocation*. The image shows the inter-chromosomal recombination that involves the sites 14q32 and 18q21 which bring to the production of der(14) and der(18) (Bakhshi, et al., 1987).

3.1.2 Probes for RNA FISH and DNA FISH

The probes used for the project are for three different targets, two for DNA sequences and one for RNA transcripts. RP5-998D24 is the probe recognising the IgH gene and it's a PAC clone (P1 artificial clones). RP11-299P2 is the DNA probe which binds to *BCL2* gene and it's a BAC (Bacterial Artificial Clones) probe. The probes originates from RPCI-5 male PAC clones library and RPCI-11 male BAC clones library, respectively. The locations on the chromosomes 14 and 18 and respective derivatives are shown in Figure 17.

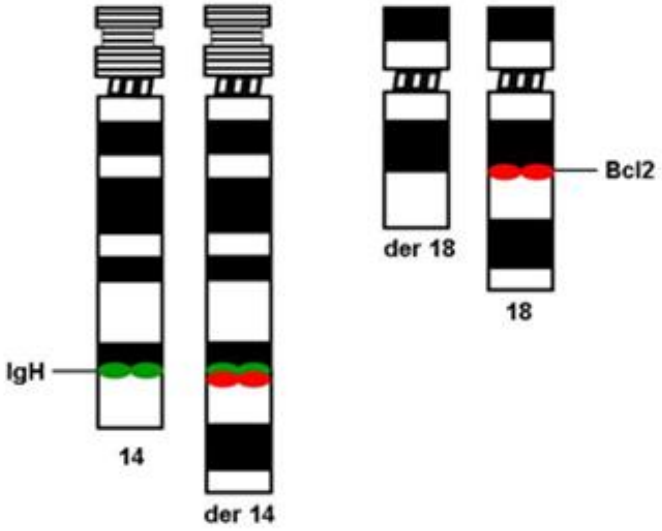


Figure 17: *The picture shows the two probes for genes detection*. IgH probe, 998D24, is labelled by FITC dye (green); Bcl2 probe, RP11-299P2 is labelled by Cy3 dye (red) (Image Garimberti's dissertation, given with permission of Dr Tosi).

The probe binding to Bcl2 transcripts was kindly given by Dr Christopher Eskiw and it recognises the nascent transcripts inside of the nuclei. The probe was labelled by biotinylated nucleotide, which binds the complex of dye-streptavidin.

3.2 METHODS

3.2.1 Cell Culture

The Pfeiffer cell line is grown in RPMI 1640 (GIBCO, Life Technologies) medium, modified with L-glutamine. 10% (v/v) of fetal bovin medium and 1% of Penicillin/Streptomycin (GIBCO, Life Technologies, 5000 U/mL) are added to the medium. The cell line is grown at 37°C in 5% CO_2 incubator till a concentration of 10^6 cells/mL. Then, it is centrifuged at 1200 rpm for 5 minutes and resuspended in 1X PBS (Sigma). The 1X PBS (phosphate buffer saline) is prepared by 1 tablet of PBS in 200 mL of DEPC-treated water. 15 ul of suspension, containing around 15.000 cells, are harvested on poly-lysine coated glass slides (Sigma-Aldrich) for a later fixation.

3.2.2 Preparation of probes for DNA FISH

Probes DNA extraction

The bacterial colonies, containing BAC and PAC clones respectively, are left to grow in Petri dishes containing LB medium added with agar (15 g/L). The LB medium (LB Broth High Salt, Miller's LB Broth) is prepared by dissolving a capsule in 1 L of distilled water, transferred in waterbath at 37°C until it is completely dissolved. Every capsule contains: Tryptone 10 g/l, yeast extract 5 g/l, sodium chloride 10 g/l, Tris/Tris HCl Buffer 1.5g/l. In order to select only the specific colony, selective antibiotic is added to LB medium. The PAC colonies are kanamycin resistant (12.5 ug/mL); otherwise, the BAC colonies are chloramphenicol resistant (12.5 ug/mL). The bacterial colonies are left to grow at 37°C for 24 hours. After that time some of them are selected and grown in tubes containing 5 mL LB medium with selective antibiotic. After 18 h in shaking incubator at 37°C 200 rpm, 2 ml of bacterial culture have been centrifuged at 3000 rpm for 10 minutes twice.

In order to extract the probes, I applied a quick alkaline lysis prep protocol. The pellet is resuspended in 300 ul of P1 buffer (15 mM Tris pH 8, 10 mM EDTA, 100 ug/mL RNase A) and then added 300 ul lysis solution, P2 buffer (0.2 M NaOH, 1% SDS) for 2 minutes mixing by inversion. Successively, 300 uL of cold 3M potassium acetate pH 5.5 are supplemented and mixed until a “snow-storm” appeared. The reaction is then incubated on ice for 10 minutes to precipitate the DNA. The next step is to centrifuge at 13000 rpm at 4°C and the supernatant

moved in a clean 2 mL tube, taking care to take only the supernatant. The last step is mixed with 700 uL ice-cold isopropanol and incubated for 30 minutes at room temperature. After incubation, the precipitate is centrifuged at 13000 rpm at 4°C for 15 minutes and the supernatant is discarded. Firstly, the pellet is washed in 500 uL ice-cold 70% ethanol and later, after the ethanol is completely evaporated, resuspended in TE buffer.

The DNA concentration and quality is checked using NANODROP analysis and electrophoresis. RP5-998D24, probe for *IGH* gene, is 2174 ng/uL and RP11-299P2 is 2460 ng/uL at NANODROP, both with good values of ratio 260/280 nm and 260/230 nm. The gel for electrophoresis is made at 1% by 0.5 g agarose in 50 mL 1X TBE (89mM Tris base, 89mM Boric acid, 2 mM EDTA), supplemented with ethidium bromide (5 mg/mL). The Figure 18 shows the bands of the two probes after the running in 1% electrophoresis gel. The sizes of both probes are too big to be enjoyed by a standard electrophoresis; the best way to visualize the right sizes of the two probes is pulsed-fields gel that it was not possible to set in our laboratory.

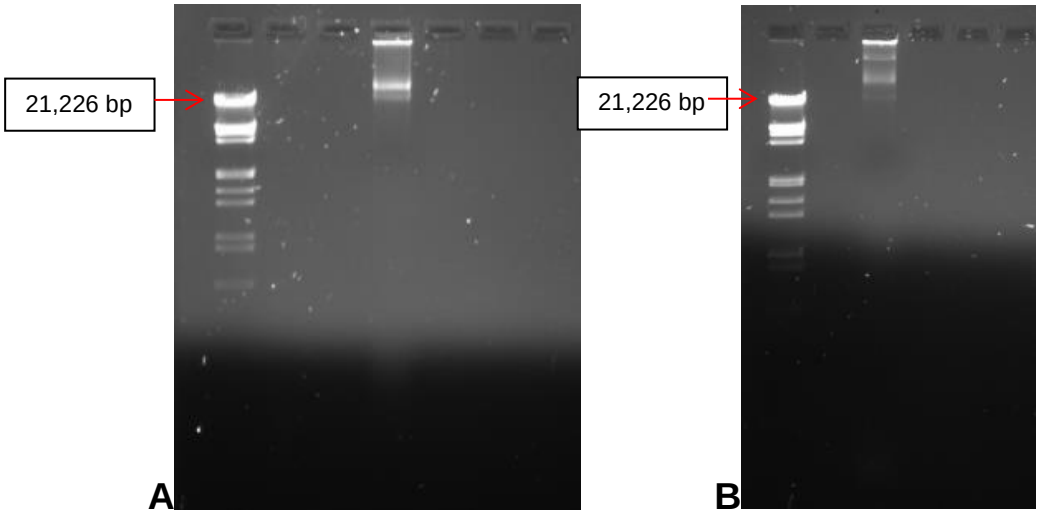


Figure 18: *1% electrophoresis gel of 998D24 and RP11-299P2*. A) the image shows the electrophoresis 1% gel after 40 minutes at 70 mV; in the first well the DNA ladder III (PeqGold), in the fourth one there is the loaded RP5-998D24 probe suspension. B) the image shows the RP11-299P2 probe suspension (third well) after running in 1% agarose gel; in the first well there is DNA ladder III to check the size of the probe. The size of RP11-299P2 was expected around 146,600 bp; the size of RP5-998D24 around 286,000 bp.

NICK TRANSLATION

The nick translation (NT) is a technique for obtaining the labelling of probes to modified nucleotide, which can be conjugated directly to a fluorescence dye or biotin/digoxigenin. To detect the probes labelled by biotin/digoxigenin is requested a second binding to a secondary antibodies or molecules labelled on its own to a fluorescent dye.

To aim the detection of the two genes, it is chosen to follow the first method to avoid cross-reacting with the probes for RNA FISH. The kit used is purchased by Abbott Molecular and includes Nick translation enzyme mix, 10x nick translation buffer, dNTP solutions and nuclease-free water. The protocol below was used for both of two probes, RP5-998D24 labelled to FITC-dUTP dye (green) and RP11-299P2 to TRITC-dUTP (red).

Protocol Nick Translation (Abbott Molecular):

- DNA sample……1 ug
- TRITC/FITC-dUTP…….0.02 mM
- dTTP……0.1 M
- dNTP mix…….0.1mM
- 10X Nick Translation Buffer…….5 uL
- Enzyme mix……5 uL
- dH_2O……up to 50 uL

The mix is prepared in ice and incubated at 15°C for 116 hours. The labelling is checked on 1% agarose gel by typical smear between 700-100 bp, depending on the size of sequences (Figure 19).

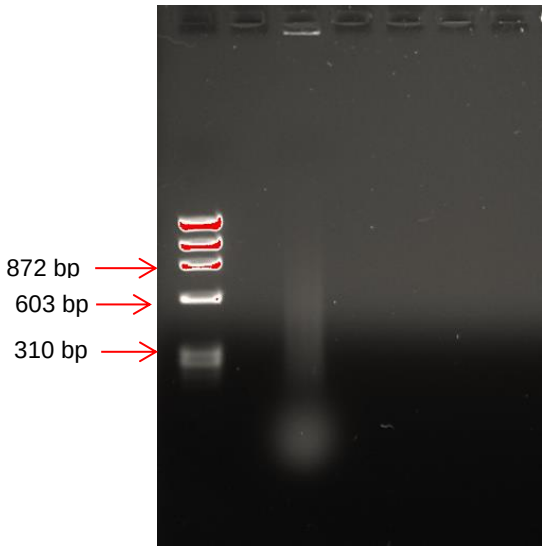


Figure 19: *Nick Translation reaction products checked in 1% agarose gel*: The image shows the running in 1% agarose gel (70 mV) of 998D24 probe after Nick Translation reaction. The DNA ladder is XIII by PeqGold.

The removal of unincorporated nucleotides is occurred by PURELINK columns (Invitrogen), supplied with following protocol. Before starting, the binding buffer (B2) is completed with addition of isopropanol and the wash buffer with ethanol. Then, the reaction is made mixing 4 volumes of B2 and 1 volume of NT product. In the second step the mix is transferred in a supplied column in 2 mL tube. After a centrifuge at 10000x*g* for 1 minute at room temperature, the eluted is discarded. The column is replaced in the same tube and 650 uL of wash buffer are added to the column. The centrifuge is done again at the same condition, 10000x*g* for 1 minute at room temperature, but twice in order to cleaning very well the column. At this point, the column is moved to another 1.5 mL supplied tube and added 50 uL of Eluition Buffer in the centre of the column. Before last centrifuge at the same condition, the tube is left at room temperature for 1 minute. The eluted is my NT mix cleaned up that I can collect at -20°C or used straight away.

After purification, it is performed the ethanol precipitation adding 10 ug of Cot-1 DNA, 1/10 of 3M potassium acetate and then 100% ice-cold ethanol was added in 2.5x (v/v). The solution is left at -20°C overnight to precipitate the DNA. The next day the suspension is been centrifuged at 10000 rpm at 4°C for 30 minutes and then the pellet is washed in 200 uL 70% ice cold ethanol. This is removed by further centrifuge at 10000 rpm at 4°C for 15 minutes. The pellet, left to dry, was

then resuspended in 5 uL of TE buffer and 45 uL of Hybridization buffer (50% formamide, 10% dextrane sulfate, 1% tween 20, 2X SSC).
The probes were stored at -20°C and ready for DNA FISH.

3.2.3 Probes for RNA FISH

The probes for RNA FISH were kindly prepared and sent by Dr Christopher Eskiw from his stock. The probes were already labelled by Biotin-dUTP and consequently they were used directly in RNA FISH.

The probes are single strands cDNA (ssDNA) made by reverse transcription from RNA template. The suggested protocol is the following: the DNA plasmid was linearised by a specific restriction enzyme. Then, it was incubated for 30 minutes at 37°C with Proteinase K to a final concentration of 100 ug/ml and SDS to a final concentration of 0.5%. It was extracted by chloroform and precipitated by ethanol. The pellet was resuspended in RNase-free water.

The successive step was *in vitro* transcription when it was prepared a mix of RNase-free water to a final volume of 100 ul, 10 ug of linearised plasmid DNA, NTP mix (25 mM each), 10x RNA polymerase buffer, RNasin and RNA polymerase (200 u of T3, T7 or SP6). The mix was incubated at 37°C for 2-4 hours and added 100 u of enzyme after 3 hours. After that, it was added 1 ul 1 mg/ml DNase I and let to incubate at 37°C for 15-20 minutes. Then, the ammonium acetate (final concentration 1.7M) is added and phenol/chloroform extract. As last step it was added 3 volumes of ethanol and let to precipitate on ice for 30 minutes. The pellet was dissolved in RNase-free water to a final concentration of 2 ug/ul.

At this point, it was prepared a reaction mix for reverse transcription where RNA template (4 ug) was mixed to RNase-free water (final volume 20 ul), random hexanucleotide mix (5-10 ug/ul) and let to denaturate at 65°C for 5 minutes and chilled quickly on ice. After that, it was added 5x Superscript first-strand buffer, 0.1 M DTT, dNTP DIG mix (2 mM each) and RNasin optionally. The mix was shacked gently and incubated for 2 minutes at 42°C. At this stage, Superscript II was added to a final concentration of 400 u. The mix was incubated at 42°C for 90 minutes.

In order to degrade the RNA template 2 ul 4 M NaOH is added to the mix and incubated for 30 minutes at 37°C. Then 2 ul 4 M HCl is added to neutralize the pH.

The pellet was purified by Qiaquick Nucleotide Removal Kit (Qiagen) or alternatively the labelled probe ethanol precipitated.

3.2.4 RNA DNA FISH

The technique performs a detection of probes for RNA and DNA simultaneously, in order to collocate the nascent transcripts with their genes. The first part of the method is to bind the probes to RNA and it's performed completely in RNase-free environment.
In order to keep the RNase-free environment, the bench was cleaned with 70% RNase-free ethanol and the poly lysine slides (Thermo Scientific) and coverslips were dunk in 70% RNase-free ethanol, then rinsed in DEPC-water. Buffers and solutions for the RNA FISH part were made with DEPC-water when requested.

Protocol:

First step – FIXATION (1st day)

The cell line is treated as explained in Cell Culture paragraph (3.2.1). Before to start, the coverslips and the poly-lysine slides are washed to eliminate any RNase traces and so they are washed in 70% RNase-free ethanol and rinsed in DEPC-water before use. 15 uL of cell suspension are dropped onto slide. The area is previously delineated by ImmedgePen (Vector Laboratories), a hydrophobic barrier which it also favours the cells to fix. The suspension is maintained for 1.5 minutes in an aseptic area. The cells are then fixed in 4% formaldehyde/5% acetic acid in 10X saline buffer (NaCl in DEPC-water) for 18 minutes at room temperature. The slide are laid flat in order to keep the mostly of the cells.

The slides are washed in 1XPBS three times at room temperature for 5 minutes each washing. Before freezing the slides, the fixation is checked at optical microscope. The slides are collected at -20°C in 70% RNase-free ethanol, at least for some days before going ahead with the technique.

Second step – HYBRIDIZATION (2nd day)

In an aseptic environment, the slides are thawed at room temperature in fresh 70% RNase-free ethanol. After that, they are washed in a new jar containing Tris-saline (TS) buffer (0.1M Tris-Cl, pH 7.5, NaCl 0.15M). The nuclei are already permeabilized in the previous day step when they were let to incubate with formaldehyde and acetic acid. Then, the nuclei are deproteinizated in 0.01% pepsin/0.01M HCl at 37°C for 5 minutes. Later, the slides are rinsed in DEPC-water and then fixed again in 3.7% formaldehyde/PBS for 5 minutes. After they are washed in 1XPBS, the nuclei are dehydrated in a serial washing of 70%, 90% and 100% ethanol for 3 minutes each at room temperature.

Contemporary, 2 ug of Biotin-labelled probes are mixed to 8 uL with RNA Hybridization mix (50% formamide, 2XSSC, 200 ng/uL of Salmon Sperm DNA (Sigma), 5X Denhardt's solution (Sigma-Aldrich), 50 mM phosphate buffer pH 7.0, 1 mM EDTA). The mix is denaturated at 80°C for 5 minutes and then it's hold in ice. The denaturated probe is presented at the nuclei previously permeabilized. The area, where the probes and the nuclei are, is covered with 22x22 mm coverslips and seal with glue. They are hybridized at 37°C in a moist chamber inside the waterbath and the reaction is left for 36 hours.

Third step – DETECTION (3rd day)

On the third day the coverslips are removed in 2XSSC, prepared with DEPC-water. Then, they are washed three times at 37°C in 2XSSC, for 5 minutes each. After that, the slides are soaked in Trissaline- Tween®20 (TST) (0.1 M Tris-Cl, 0.15 M NaCl, 0.05% Tween®20) at room temperature for 5 minutes.

In order to detect the probes and their targets, it's added 100 uL of Tris-saline-Blocking (TSB) solution (0.1 M Tris-Cl, 0.15 M NaCl, Blocking Reagent (Roche)) on each slide. The blocking is 1.35% in TS. The slides are left to incubate covered by parafilm in a moist chamber at room temperature. The humidified chamber is prepared soaking a paper in TS Buffer. The first molecule added for the detection is Avidin-Cy5. The stock is diluted 1:400 in 100 uL total of TSB and poured onto slide. It is left to incubate in moist chamber for 30 minutes at room temperature, after it is covered with parafilm. The next steps are done in the dark, according to

the light sensitive fluorochrome used. The slides are washed with TST Buffer twice for 5 minutes each at room temperature. After that, the anti-Avidin-Biotin stock is diluted 1:200 in 100 uL of TSB Buffer and added onto slide. The incubation is made at the same condition for the same time long. Once finished the time, the slides are washed again in TST Buffer for 5 minutes each washing at room temperature. The last step is the supplement of Avidin-Cy5, diluted in the same way above said. The slides are incubated at room temperature in moist chamber for 30 minutes and later they are washed in TST Buffer for 5 minutes a couple of times, before to get in DNA FISH.

Fourth step – DNA FISH HYBRIDIZATION (3rd day)

The same day, the DNA FISH is started with one washing in 1XPBS and then post-fixated in 3.7 % formaldehyde/PBS at room temperature for 5 minutes. The slides are rinsed in 1XPBS and then transferred in a jar containing 2XSSC for 5 minutes at room temperature. The denaturation of the nuclei is made at 73°C in 70% formammide/2XSSC for 5 minutes. The DNA probes are prepared mixing 1 ug from each DNA probe (one is for IgH gene targeting and the second one is for Bcl2 gene targeting) with DNA Hybridization mix (50% formamide, 10% dextrane sulfate, 1% Tween®20, 2X SSC) up to 8 uL for each slide. The probes are denaturated at 72°C for 5 minutes and then at 37°C for 20 minutes. Before the hybridization, the slides are washed in ice-cold PBS three times for 5 minutes each and let to air dry. At the end, the denaturated probes are presented onto slides, the area is covered with a coverslip 22x22 mm and seal with glue. The hybridization is made overnight long at 37°C in a moist chamber.

Fifth step – WASHING (4th day)

The last steps are serial washings in order to clean up the slides from the probes not binding the targets. The first one is at room temperature in 2XSSC as long as the coverslips are removed (around 5 minutes). The second washing is at 65°C in 0.4XSSC for 5 minutes. The third one is at room temperature in 4XSSC/0.05% Tween®20 for 5 minutes and the last one is in 1XPBS for 5 minutes at room temperature.

The last step is for staining the nuclei and it is used 15 uL of DAPI (0.05 µg/mL) on each slides. All the area is then covered with coverslip 22X40 mm and seal with glue. Once dried, the slides are left at +4°C for some days before the analysis at fluorescence microscope, because I observed that the fluorescent dyes were clearer if left in the fridge longer.

After this protocol, the slides are analysed at fluorescence microscope HF14 Leica DM4000 SOP v2, equipped with automatic wheel, which contains the proper filter sets that will allow excitation of the dye and capture of fluorescence. The images are taken by CCD camera with 100x magnify and adjusted by Leica AF6000 software. Beside, considering that the nuclei hold a 3D structure, every channel is taken at several focal planes for detecting all the signals for one nucleus at time.
All the images taken in one nucleus are then overlaid in order to create a manual merge image, which is the composition of single channels and focal planes.
Below (Table 2), the characteristic wavelengths of emission and adsorption of every dye used in the project are listed.

Name	Emission	Adsorption	Colour
DAPI	350	456	Blue
FITC	490	520	Green
TRITC	595	615	Red
Cy5	685	650	Purple

Table 2: *Table of emission and adsorption of dyes.* The table shows the wavelength of emission and adsorption of every dye used for the project.

Chapter 4

RESULTS

4.1 Results

The project aims to understand the origin of the BCL2 transcripts inside the Pfeiffer cell line nuclei that is characterized from the translocation t(14;18), typical of the follicular lymphoma cells. In other words, by the technique of RNA DNA FISH we see the signals from transcripts along with *IGH* and *BCL2* genes which are involved in the chromosomal translocation t(14;18). RNA DNA FISH experiment is a technique which allows visualizing genes and transcripts simultaneously. The procedure has been used to localize RNA and DNA in the nucleus for being able to improve the knowledge of the nuclear organisation of genes and their transcripts. In this specific case it was decided to use this experiment for analysing possible changes in the nuclear arrangement because of the presence of t(14;18) chromosome.

I repeated the experiment to adjust the final yield three times. In the last experiment, as described in Material and Methods, I counted 118 visible nuclei with signals. The nuclei with fusion are 93 on 118 counted; that it means the 78.8% of the analysed Pfeiffer cells show the *IGH/BCL2* translocation and among these nuclei the 61% has the Bcl2 transcripts from the fusion. The total number of analysed nuclei (118) is then subdivided in two different populations: the first has the fusion signals and the other one not (Table 3).

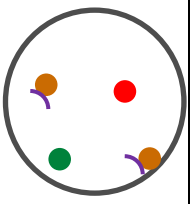
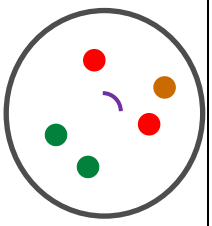
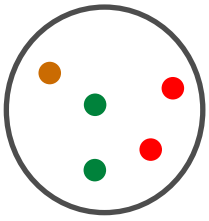
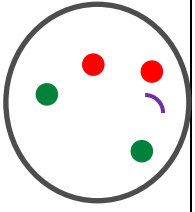
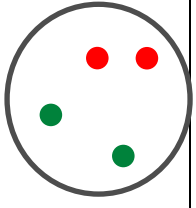
Nuclei with fusion gene			**Nuclei without fusion gene**		Legend
RNA signals from fusion	**RNA signals from out of the fusion**	**No RNA signals**	**RNA signals**	**No RNA signals**	
Ia	IIa	IIIa	Ib	IIb	= *Bcl2* gene signal = IgH gene signal = fusion signal = Bcl2 transcripts signal
					
61%	14.4%	3.4%	12.7%	8.5%	
78.8%			20.2%		

Table 3*: The subpopulations of analysed Pfeiffer cell line nuclei*. The table shows the different types of nuclei based on the origin and position of Bcl2 transcripts. The first group show the nuclei with fusion gene characterizing the 78.8% of the total nuclei; the 21.2% is the percentage of the nuclei without fusion signal. In turn, the first one is subdivided in: Ia) 61% nuclei with fusion and transcripts from translocations; IIa) 14.4% nuclei with fusion and RNA signals from other sites in the nucleus; IIIa) 3.4% nuclei with fusion gene but no transcripts. In the second group the population is divided in: Ib) 12.7% nuclei no fusion gene but with Bcl2 transcripts; IIb) 8.5% nuclei no IgH/Bcl2 translocation and no transcripts.

In turn, the group characterized by fusion gene is classified in three subpopulations, based on the origin and presence of transcripts signals: I) Bcl2 transcripts from fusion; II) Bcl2 transcripts in other sites of the nucleus; III) no Bcl2 transcripts inside of the nuclei with fusion.

The most abundant group is the first one where I counted that in 72/118 nuclei (61% over the total analysed nuclei) it's possible to see clearly the origin of the transcripts from the *IGH/BCL2* translocation (Figure 20).

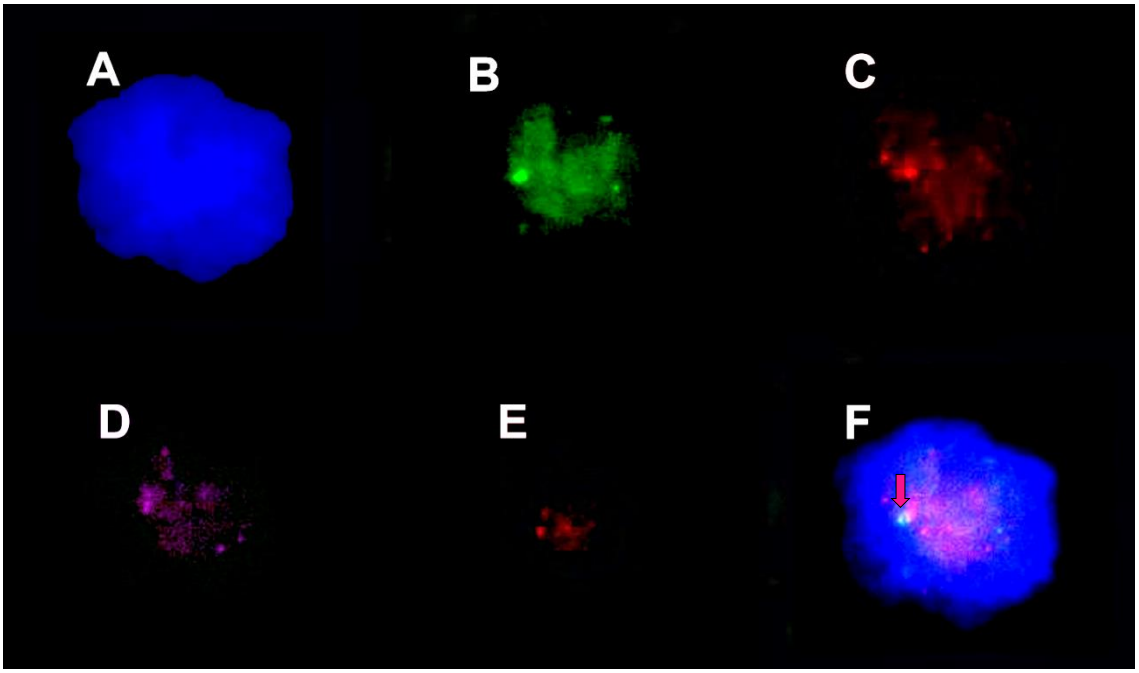


Figure 20: *Population of Pfeiffer cell line with transcripts originating from IGH/BCL2 translocation*. The first four images represent the same nucleus on the same focal plane: A) DAPI staining; B) FITC (green) channel, *IGH* gene; C) Cy3 (red) channel, *BCL2* gene; D) Cy5 (purple) channel, RNA signals. E) It represents another focal plane of Cy3 channel

where I found *BCL2* gene signals from other locations. F) The Merge image of every channels and different focal planes where I detected the signals. The pink arrow in the Merge image points where the fusion signal is along with its transcript.

The image shows the most cases found in Pfeiffer cell line. The signals of transcripts are commonly characterized by elongated shape, even if sometimes the strong signals and the noise of background let them appear as dots. In the Figure 20 it's possible to count two green signals from *IGH* gene and two from *BCL2* gene labelled by Cy3 (red). In addition, I consider that the other two purple spots are real transcripts signals, because it is seen in many of the analysed nuclei of Pfeiffer cell line.

Nonetheless, there are different shades belonging to this group, due to the presence of two or more copies of the fusion gene. 30/118 cases evidence only one translocation and one or multiple signals from single genes, but there are further 18 cases where it's observed two fusion genes signals and 13 nuclei where the signals of the fusion are even three or four. In these last cases the nascent transcripts originates from at least one of the copies or more.

In figure 21 it is shown a case where two signals from fusion gene are seen and one single green signal and one single red are present too. The purple dots from RNA transcripts originate from both of the fusion signals. There is one bright purple spot which looks like originating from red single signal. The cause could come from a background spot or a real signal, but since it is the only case observed is not possible to explain it. The observation of the shape of the spots coming from fusion genes is more elongated, as the transcripts typically appear by FISH experiment. However, it's not possible to confirm the result of this image

because of lack of control.

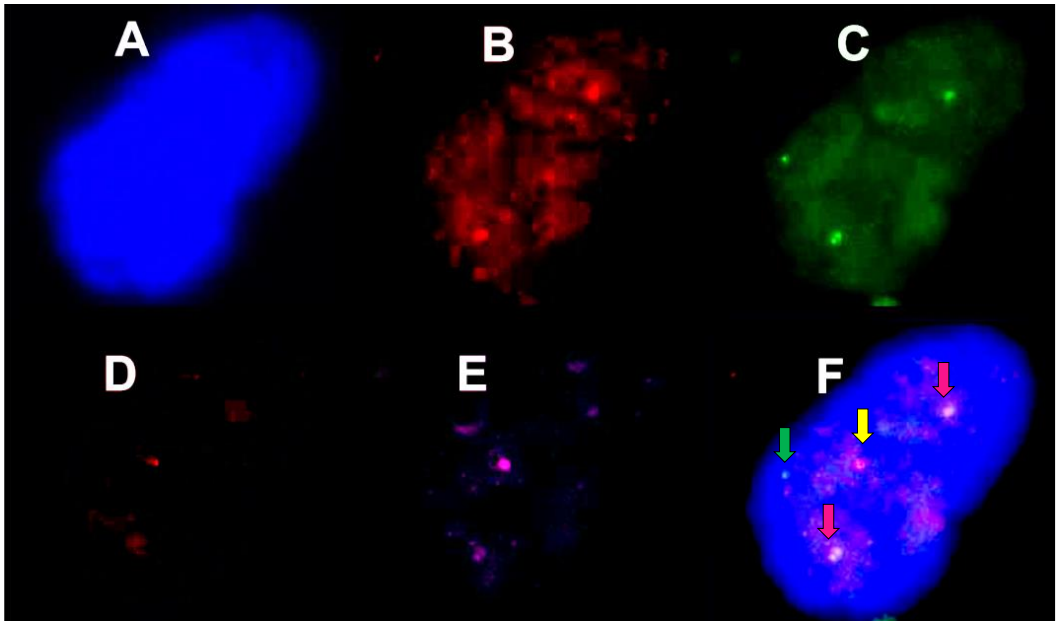


Figure 21: *Nucleus with Bcl2 transcripts from fusion and single gene*. The first row shows the images taken from the same focal plane: A) DAPI staining; B) Cy3 channel, two signals of *BCL2* gene; C) FITC channel, three signals of IgH gene. In the second row: D) and E) are taken in the same focal plane, D) has one *BCL2* signal (red); E) has three signals of Bcl2 transcripts, where the brightest originates from the single gene of the different focal plane. F) It is the Merge image of the previous ones. The two pink arrows point where the two fusion signals are along with their transcripts; the green arrow points the single *IGH* signal and the yellow arrow points where the single *BCL2* signal is seen along with a bright purple dot.

The nuclei with transcripts not originating from fusion genes belong to the second subgroup. The detected signals of RNA are spread inside of the nucleus but they don't come out from specific genes, even if they are in the proximity of *BCL2* single gene or the fusion gene (Figure 22).

The smallest group counts only 4/118 nuclei. In this one the *IGH/BCL2* genes are detected, but no Bcl2 transcripts signals are found.

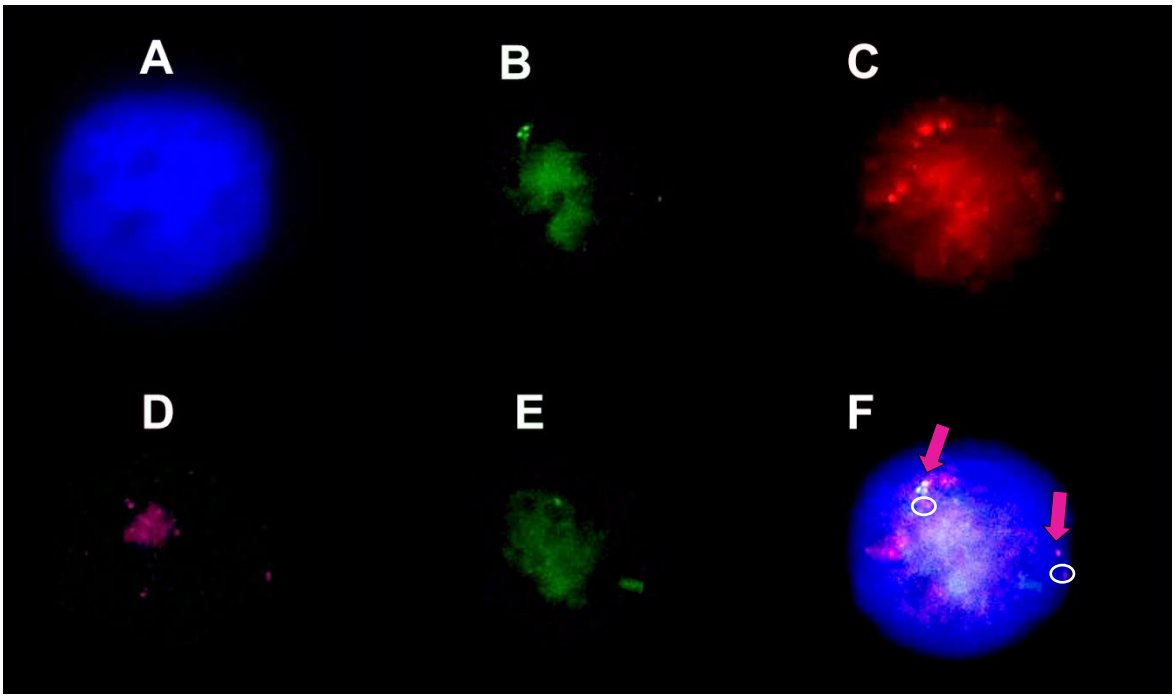


Figure 22: *Nuclei with Bcl2transcripts not originating from fusion*. The first four images are the four fluorochromes detected in the same focal plane: A) DAPI staining; B) FITC channel, where it's possible to count four signals of IgH gene; C) Cy3 channel, where I count four signals of Bcl2 gene; D) Cy5 channel, four transcripts signals. E) FITC channel taken from a different focal plane. F) the Merge image of overlaid channels. The pink arrows point the two fusion signals; the two white circles localize the two transcripts signals. The RNA signals are close to the fusion genes but not from the same origin.

25/118 analysed nuclei represent the second population. Although they haven't the signal of fusion gene, some of them show the expression of *BCL2*. The percentage of the nuclei with the expressed *BCL2* is of 12.7% and their signals are seen spread in the nucleus. However, only in few cases it's possible to appreciate that the Bcl2 transcripts originate from the *BCL2* gene or they are found next to that (Figure 23).

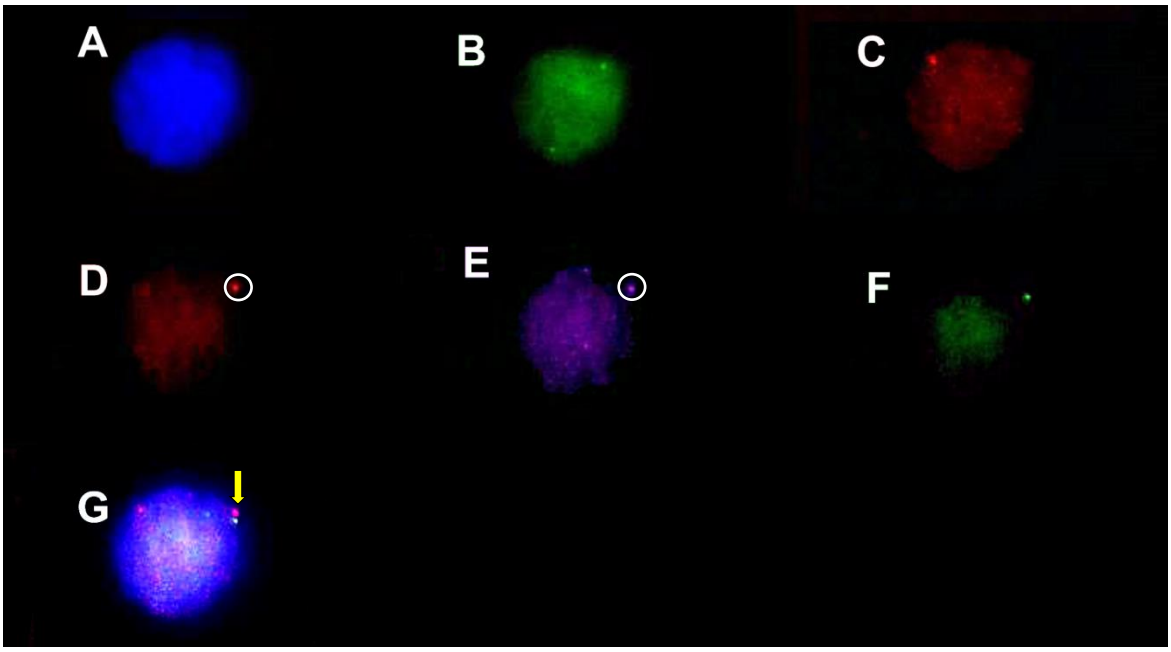


Figure 23: *Image of nucleus characterized from Bcl2 transcript originating from BCL2 gene*. In the first row, three channels are detected from the same focal plane: A) DAPI staining; B) FITC channel, two signals of IgH gene; C) Cy3 channel, one signal of *BCL2* gene. In addition, I took three different channels from another focal plane, shown in the second row: D) Cy3 channel, one signal of *BCL2* gene; E) Cy5 channel, three signals of Bcl2 transcripts where one results brighter than the others; F) FITC channel, one signal of IgH gene. In the last row (G), it's shown the Merge image of the previous images. In figures D and E the white circle localizes the *BCL2* gene and the BCL2 transcript, respectively. The yellow arrow in figure G points the *BCL2* signal along with its transcript.

The Figure 23 shows the case where the nucleus has the *BCL2* and *IgH* genes really close one to each other and the brightest RNA signal is over and coincident with one of the Cy3 (red) signal. Both of the *BCL2* gene signals are in peripheral localization of the nucleus.

8.5% of the nuclei represent those without translocations and transcripts signals. According to what I showed so far, the experimental procedure that I used localized some active gene in a peripheral position.

I observed that 40 out of 72 nuclei with chromosomal translocation and transcripts had the t(14;18) in a more external position. My observation should be supported by analysis of a control cell line and by analysis by specific software. From the visual analysis, I considered as external the signals taken in the focus plane closer

to the observer and the signals at the edge space of the nucleus. Most of the times the signal of t(14;18) is clearly at the edge of the nucleus (Figure 24).

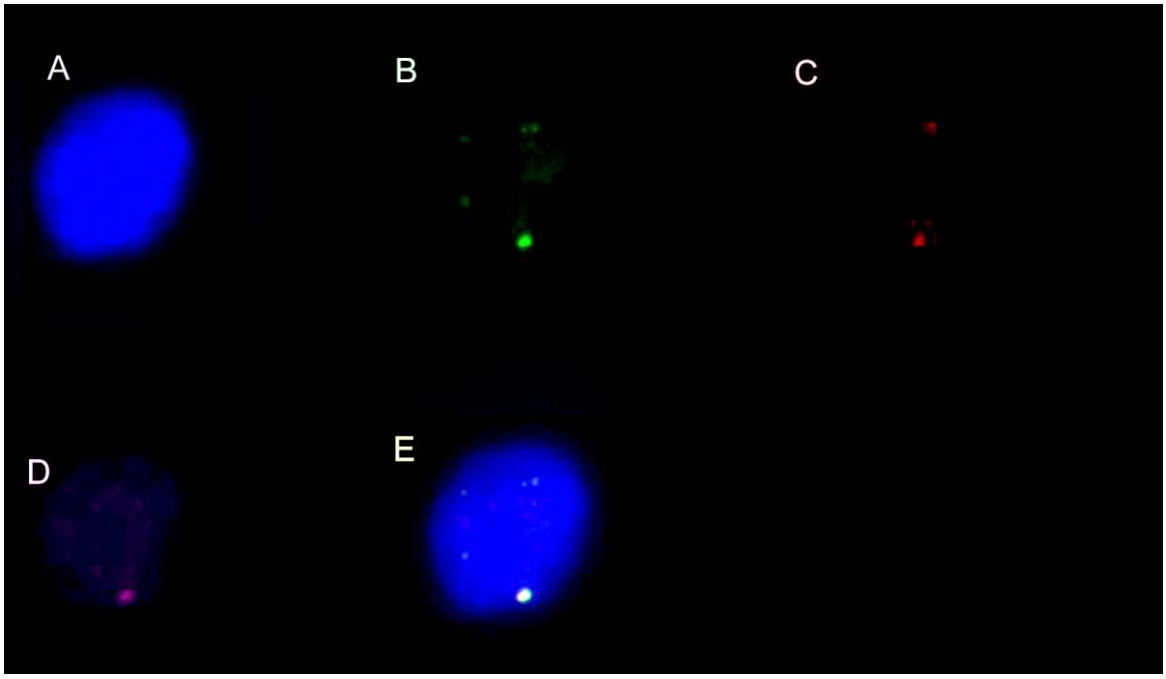


Figure 24: *t(14;18) in the periphery of nucleus*. A) It shows the DAPI staining nucleus; B) It is the FITC channel (IgH gene). It shows the strong signal at right bottom from translocation; C) It shows the TRITC channel (*BCL2* gene) and the strongest signal at the right bottom is from the t(14;18); D) It is Cy5 channel and it shows the origin of the Bcl2 nascent transcript; E) It is the merge image, where all the channels are combined.

Contrarily to what Garimberti's work hypothesized, it looks like that the t(14;18) has not a preferential position to be activated. However, I found that the bimodal distribution of the chromosomal translocation that she showed in her unpublished work is clear also in the images taken in my experiment. Some pictures show two or more signals due to translocation and all of them have the transcripts originating from there. Although they are all transcribing the Bcl2 mRNA, the nuclear localizations of them looks like in a peripheral nucleus in more than half of the analysed nuclei. Otherwise, I find that a preferential localization is common for all the t(14;18) copies inside of the same nucleus. Every time I found two or more t(14;18) activated along with their transcripts inside of the same nucleus, all of them are at the periphery or in a more central localizations. Probably the

preferential nuclear position of the active gene depends by stage of differentiation of the subpopulation of the Pfeiffer cell line.

Chapter 5

DISCUSSION

5.1 Discussion of the results and future experiments

The translocation t(14;18) is a chromosomal aberration found in around 80% of follicular lymphomas and 30% of diffuse large B-cells lymphomas (Lin & Medeiros, 2007). In this translocation, the *BCL2* gene is moved next to the *IGH* gene. The change in position leads to the overexpression of *BCL2*. The aim of my project was to visualize the origin of the BCL2 transcripts in the nucleus and to see if the their origin was from the same localisation as the t(14;18) chromosome. If the result was positive, this could prove that the chromosomal translocation is the responsible of the overexpression of *BCL2*.

The Pfeiffer cell line is characterised by the presence of the chromosomal rearrangement t(14;18). From my experiments, the detection of the *IGH/BCL2* fusion gene has been found in 80% of the nuclei analysed. I expected that around 90-100% nuclei should have the translocation using in situ hybridisation. However, this difference in percentage of positive t(14;18) might be accounted for the evolution of the cell line *in vitro*.. Data not shown confirm that Pfeiffer cell line can evolve and it can show new chromosomal translocations and loss of some signal from t(14;18). Moreover, usually the efficiency of the fluorescence in situ hybridisation is usually less than 100%. During the development of the experiment, I experienced some issues in visualizing the signals from genes due to non specific background signal so I made adjustments to reduce the background thereby improving the efficiency of the hybridization.

My analysis made on the images taken show that 23 out of 118 nuclei analysed have more than two signals from the fusion. The result could be due to two reasons: first, the translocated gene was amplified leading to an increased expression of *BCL2*; secondly, the result could be altered by the presence of background. The comparison among all the images analysed supports that the second option might not be true. The signals from background were mainly characterized by single dots shape and furthermore when the signals were overlapped they looked like smaller than the real signals. Considering this, I can speculate that a subpopulation of the cell line shows more than one copy of the translocated gene. The consequence could be an increased expression of *BCL2* compared to other DLBCL cell lines. It might be interesting to quantify the expressed *BCL2* in two different cell lines featured by t(14;18) and to see if the

difference of the expression could be dependent by the presence of duplication of t(14;18).

Previous work performed in Dr Tosi's lab (Garimberti & al, 2009), which my project comes from, was discussed in the first chapter of this thesis. Elisa Garimberti did 2D FISH procedure to study the localization of *IGH* gene, *BCL2* gene in their respective chromosome territories, 14 and 18. The project used Pfeiffer cell line as a model, which is characterized by the chromosomal translocation t(14;18), involving gene *IGH* and *BCL2*. The aberration leads to formation of fusion gene which is responsible of the overexpression of *BCL2.* The work showed a percentage of nuclei with translocation similar to my result, without observing the presence of more copies of chromosomal translocation in a subpopulation of the Pfeiffer cell line.

I didn't find any literatures that could support what I observed in the nuclei of Pfeiffer. Some previous work observed a high expression of *BCL2* in cells without translocation t(14;18). They observed in patients with DLBCL there was overexpression of *BCL2* due to gain or amplification of 18q where the *BCL2* gene is. Their observation theorizes that in order to develop the lymphoma cells need to overexpress the anti-apoptotic protein, even when the t(14;18) wasn't evident (Monni, et al., 1997), (Leich, et al., 2009). It would be interesting to study if the expression of *BCL2* in different stage of proliferation and cell cycles changes and increases based on different necessities of the cells. It could be useful to test different DLBCL cell lines under different situations and to figure out if there is a mechanism which actives the amplification of *BCL2* or t(14;18) leading to overexpression of the anti-apoptotic protein.

A further consideration on the expression of *BCL2* is about the observation that the origin of the nascent transcripts of BCL2 is seen also in the proximity of *BCL2* gene. The cases to describe this opinion are those shown in Result section where one nascent transcript signal originated from *BCL2* single gene (Figure 23). Considering the results, it's clear that the protection from apoptosis in Pfeiffer cell line is an important step to maintain tumour development. The signals of BCL2 RNA are not found just from the same origin of t(14;18) or amplified *BCL2* gene but it's detectable also in other sites inside of the nucleus and can occur, close to

or far away from the genes. This could mean that *BCL2* is transcribed frequently in a single cell, even without the translocation. It is right to consider that some spots could be part of the background, even if I selected those based on the observation of many analysed nuclei.

Previous studies demonstrated that the overexpression of BCL2 is very important for the development of some lymphomas. The dysregulation of apoptosis due also to BCL2 overexpression plays an important role in the development of lymphoma cells (Ott & Rosenwald, 2008). Its overexpression might be important as a molecular target for fighting the resistance of tumour cells to chemotherapy as it's anti apoptotic function can be targeted for therapy (Thompson, et al., 2013).

One of the findings from Garimberti's work was that in 15% of nuclei analysed the *BCL2* gene loops out from its territory to a central position in the nucleus. By FISH experiments, it was demonstrated that the looping out *BCL2* gene was always the *BCL2* gene involved in the translocation. Consequently, Elisa Garimberti hypothesized that only this gene could be transcriptionally active. The hypothesis was that this position favoured transcriptional activity, according to that the activation of genes is also dependent from looping out of the gene from their chromosome territories (Volpi, et al., 2000). I found out in my project that the nascent transcripts were not only originated from t(14;18), but also from *BCL2* single gene and I also observed that the transcriptionally active fusion gene was always not in a central position.

Related to what I observed, a further result of the previous work was a bimodal distribution of t(14;18) related to the positions of analysed genes. The authors speculated that this could be caused by gene expression. In other words, the interior nuclear position of chromosomal translocation reflected activation of gene.

I tested the hypothesis detecting the *BCL2* and *IGH* genes and the transcripts of *BCL2*, in order to find if the origin of RNA was from the translocation and if the nuclear localization of the transcriptional active gene was central. According to many previous studies, the periphery of the nuclei is the "dark side", in other words where the transcription is less active (Deniaud & Bickmore, 2009). Many studies highlighted that the gene-poor chromosomes were preferentially at the periphery of the nucleus. Based on these findings, Garimberti et al. speculated that the

position of t(14;18) in the centre of nucleus was responsible of the overexpression of BCL2.

It's known that the chromosome positioning has a non-random organization within the cell nucleus. Many genes move from central position to the periphery when they need to be inactivated. The change in positioning appears to be the cause of the silencing of these genes, because there are proteins connected to the nuclear membrane responsible for inactivation. The silencing of transcription in the nuclear periphery is firstly due to binding with SIR (Silent Regulation Information) proteins that are able to condense the chromatin and also binding with the nuclear lamina which regulates the activation of chromatin. However, at nuclear membrane there are also nuclear pore complexes (NPC) that mediate the passage of the mRNA from interior to exterior of nucleus (Kalverda, et al., 2008). Hence, Ragoczy et al. claimed a peripheral position at early stage of the transcription of β-globin and only later it moved to interior localization (Ragoczy, et al., 2006) as if this was the consequence of transcription and not the cause.

These previous findings could explain the different localization of activated genes in RNA DNA FISH experiment performed for this project. In my experiment, the results could be related with what Kalverda and colleagues claimed. The peripheral localization of some genes is probably due to a first robust transcription when it's requested a high production of the protein and consequently high passage of the mRNA in the cytoplasm.

Obviously, this theory has to be supported by further analysis. It could be useful to find the evidence of what I just said with a technique as RNA-DNA FISH coupling with immuno-FISH. The method could find if the active genes coupling with their nascent transcripts are in the periphery because of the bound with nucleoporins or other proteins that they could recruit the activated genes at the periphery.

Another future experiment that I suggest is to treat Pfeiffer cell line with inhibitors of expression of antiapoptotic protein *BCL2* and detect if the nuclear position is affected by the change of gene expression. An example of a treatment to induce a decrease of BCL2 protein is by antisense oligonucleotides (such as Genasense)

(Loomis, et al., 2003). The antisense therapy is used to inhibit the expression of specific genes. The antisense oligonucleotide is complementary to the mRNA; and binds to the transcript thereby inactivating it.

A further interesting article about how to reduce the level of *BCL2* expression is based on the correlation between the expression of *BCL2* and *BACH2*, a transcriptional repressor. The authorsdeclare that the expression of *BCL2* is significantly reduced in those DLBCL patients with an high expression of *BACH2* (Green, et al., 2009). It could be nice to see if in a DLBCL cell line is present a significant relation between the expression of *BCL2* from t(14;18) and the transcriptional repressor.

My project was set up to see the origin of the *BCL2* transcripts is from t(14;18). In the nuclei without signal from chromosomal translocation the expression of *BCL2* is still active. This might demonstrate that the overexpression of *BCL2* in the lymphoma cell line is due to activity of *IGH/BCL2* and *BCL2* single gene.

In Elisa Garimberti project, she hypothesized that the fusion gene from the chromosomal translocation t(14;18) was solely responsible of the transcription of *BCL2* in Pfeiffer cell line. As I showed in my results, the expression of BCL2 in Pfeiffer cell line also originates next to non-translocated *BCL2* . The finding is further supported by 12.7% of nuclei with the presence of RNA signals from the nuclei with a lack of fusion signals where there are BCL2 transcripts, signals from the *BCL2* gene and *IGH* gene. It could be possible that in the same nucleus the transcripts are made by *IGH/BCL2* and *BCL2* gene simultaneously. The lack of control experiment limits investigating this further. Figure 21 shows an example where two fusion genes are the signals in the same origin of the transcripts signals. The two active genes are more in a peripheral localization, but a brighter purple dot looks like from the origin of *BCL2* single gene. The quality of the image doesn’t allow me to say if it is a real signal or from non-specific background. However, it could be nice to repeat the experiment with other lymphoma cells characterized by t(14;18) and to see if it happens as well.In conclusion, I can summarize that my findings confirm the presence of the t(14;18) in the most nuclei of Pfeiffer, which is correlated with an elevated gene expression of *BCL2*. Even if it’s clear the fusion gene is widespread, it’s not seen in every nucleus but only in

78.8%. This could be referred to possible mutations present in the Pfeiffer cell line. It could be interesting to study which alterations happened in the mutated cell line.

Further finding shows that the production of BCL2 mRNA is not only due to the translocated gene, as Garimberti's work postulated but it's supported also by the activation of *BCL2* gene, even in absence of t(14; 18) this is supported by 12.7% of the cases where a robust expression of *BCL2* occurs without translocation and, also in presence of t(14;18) the nascent transcript signals is found from the same location or in proximity of *BCL2* gene.

At a first look it seems that the BCL2 RNA is positioned at the periphery of the nucleus in many cases. One of my suggestions for future experiments is to prove and define my findings by a more careful analysis using appropriate software to define the positioning of FISH signals in the nucleus along with gene expression in a three dimensional view.

APPENDIX

METHOD USED BUT NOT APPLIED

3D DNA FISH

The cells of Pfeiffer are resuspended in 1 mL of medium in a concentration of 825 x 10^3 cells/mL. 50 uL of the supension are harvested on poly-lysine coated slides (around 40000 cells/mL) and then they are left in in the incubator at 37°C and 5% CO_2. The slides are collected in quadrichamber..

First step – FIXATION

The slides are washed in 1XPBS supplemented with Ca^{2+} and Mg^{2+}. The final concentration of CaCl is 0.90 mM and of MgCl is 0.49 mM and then adjusted at pH 7.4. The fixation occurs in 4% PFA/1XPBS for 15 minutes at room temperature. The slides are laid flat in a small tank, dunk in the fixative solution. The slides are washed in 1XPBS twice for 5 minutes each at room temperature and then incubated for 20 minutes at room temperature in 0.5% triton-X-100/0.05% saponin/1XPBS (To prepare 100 mL: 500 uL of Triton-X-100 and 0.5 g of saponin in 1XPBS). After that, they are washed again in 1XPBS twice for 5 minutes each at room temperature. Later, they are incubated for 30 minutes at room temperature in 20% glycerol/H_2O. The next step is to dunk once in a specific tank freezing-resistance filled with liquid nitrogen. Once done, the slides are transferred in specific boxes which are soaked in turn before collecting in -80°C.

Second day – HYBRIDAZATION

The slides are thawed on paper at room temperature and then transfer in a jar containing 20% glycerol/H_2O for 1 minute at room temperature. After that, they are dunk once in liquid nitrogen and left to thaw on a paper at room temperature. They are again transferred in 20% glycerol/H_2O for 1 minute at room temperature and washed in 1XPBS three times for 10 minutes each at room temperature. The slides are treated in 0.1 N HCl for depurination for 5 minutes at room temperature. Then, they are washed in 2XSSC three times for 5 minutes each at room temperature. In the same time, the mix of probes of Bcl2 and IgH genes (around 2 ug from each) is denaturated at 72°C for 5 minutes and at 37°C for 30 minutes. When it left only 5 minutes at the end of denaturation, the slides are transferred in 70% formamide/2XSSC at 73°C for 3 minutes exactly and then in 50%

formamide/2XSSC at 73°C for 1 minute exactly. Once probes and slides are denaturated, 8 uL od probe suspension is present on each slide. Then, the slides are covered with coverslips 22X22 mm. The coverslips are sealed on the slides and these latter ones are incubated at 37°C overnight in a moist chamber.

Third day – WASHING

The slides are taken off from the chamber and transferred in 2XSSC for 5 minutes shacking at room temperature, in order to remove the coverslips. When I ensure that the coverslips are off, the slides are transferred in 0.4XSSC at 70°C for 4 minutes and then in 4XSSC/0.05% Tween®20 at room temperature for 5 minutes. The last washing before adding DAPI stain is in 1XPBS for 5 minutes at room temperature. Once the staining of nuclei is done, the slides are sealed with coverslips 22X40 mm and collected at +4°C in a dark box to protect them from the light.

ANALYSIS

The images are taken by fluorescence microscope HF02 Axiovert 200M (Cell Observer) SOP v1.The equipment of the microscope is composed with Zeiss Axiovert 200M. The images are set and edited with AxioVision 4.6.3 software. The Cell Observer is a microscope able to combine multichannel together. Its filters are for FITC, Cy3, DAPI and Cy5. Furthermore, the difference with other fluorescence microscope used is that it's possible to take a combination of images of the same object from different focus planes. The method is called z-stacking and the user can set and modify the different layer from every focus plane. The user can also set the distance between one layer and next one and the setting of the planes can be adjusted for every channel.

Result to 3D DNA FISH

At the beginning, the experiment was thought to be developed by the 3D DNA FISH. The technique is one of the most used for figuring out better the spatial nuclear organization and how this influences the gene expression.

I had tried different approaches for several times, without good results. The images taken showed at first a poor concentration of cells, and it was barely

possible to visualize the signals in the nuclei. So I tried to better the fixation of the cells performing as following: I) incubate the slides with medium suspension longer (around 2 hours) and arising the concentration of cells in suspension; II) dunk more times the slides in liquid nitrogen. Both of the efforts didn't bring improvements. The nuclei appeared damaged and the number of them was still low.

The decision to abandon the choice of 3D DNA FISH was also dependent by obtaining a 3D view using the RNA DNA FISH protocol. This result wasn't expected and I think it was based on the gentle way to fix the cells in the method.

The images from 3D DNA FISH were taken at Cell Observer that it's a microscope able to taken the pictures with "z-stack method". The technique consists to combine multiple images of the same object from different focus planes. The name derives by the z axis along which the several images are taken.

The images of the results are not shown because of the poor quality.

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