Characterization of secondary malignancies in patients diagnosed with Chronic Lymphocytic Leukemia

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01-04-2021

ABSTRACT

Chronic lymphocytic leukemia (CLL) is the most frequent type of leukemia in the Western world. CLL can be distinguished by the presence of a clonal expansion of $CD5^{+}CD23^{+}$ B-cells in the bone marrow, blood, and secondary lymphoid tissue. Important in the pathogenesis of CLL is the activation of CLL cells, which has a B-cell receptor (BCR) gene signature. Moreover, there is also an important role for immune dysregulation in the pathogenesis of CLL. Additionally, there was also shown that CLL patients have a high level of genetic mutability and some of these mutations even have prognostic value.

There is an enhanced prevalence of secondary malignancies in CLL patients which include mostly skin malignancies, but also other solid tumors and hematological malignancies occur. The origin of a secondary malignancy in CLL patients seems to be multifactorial, including shared genetic and environmental risk factors as well as a dysregulated immune system. The aim of this study is to elaborate a hypothesis to explain biologically the association between CLL and secondary malignancies using clinical data from CLL patients with secondary malignancies. Therefore, the patient data base of the University Medical Centre Groningen was reviewed and patients diagnosed with CLL and a secondary malignancy were selected. From these 84 patients, variables were collected about patient characteristics, CLL and the secondary malignancy. Thereafter, the data was organized and analyzed. Results showed that there were 49 patients (58,3%) who carried genetic aberrations in their CLL cells. The most common genetic aberrations were del(13q14), trisomy 12, del(11q23) (ATM) and del(17p13). Results also showed that there were 25 patients (29,8%) who developed a secondary malignancy after having received treatment, in the form of systemic therapy, for their CLL. However, there were also 56 patients (66,7%) who developed a secondary malignancy without having received treatment or prior receiving treatment.

In order to test the influence of immune dysregulation and chronic BCR signaling on the development of a secondary malignancy in CLL patients, future research is needed. The influence of

immune dysregulation can be tested by looking at whether there is a difference in immune dysregulation in CLL patients with a secondary malignancy and CLL patients without a secondary malignancy. This can be done by using two 15-color antibody panels for flow cytometry. Using these panels, there is the ability to obtain a broad range of information about the patient's peripheral blood mononuclear cells (PBMCs). Both, information regarding the phenotype and the functionality of the identified subsets. There was stated that the information for each cell population covers maturation and activation, migration, responsiveness to cytokine signaling, as well as direct cellular interactions. Another technique that can be used is single cell RNA-sequencing. The influence of chronic BCR signaling can be tested by looking at whether the amount of chronic BCR signaling is the same in CLL patients with a secondary malignancy as in CLL patients without a secondary malignancy. This can be done by looking at the amount of proliferation of the cells using a CFSE-based assay, since proliferation is one of the results from BCR signaling. Moreover, the activity of both PI3K and NF-κB can also be studied.

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) can be distinguished by the presence of a clonal expansion of $CD5^{+}CD23^{+}$ B-cells in the bone marrow, blood, and secondary lymphoid tissue[1]. CLL is the most frequent kind of leukemia in Western countries[2]. For example, in the United States in 2016, CLL was believed to be responsible for ~19,000 of all the cancers that were discovered. Moreover, it is also characteristic for CLL that men have a two-times higher probability to develop CLL than women, and this chance to develop CLL grows with an increasing age. This is also shown in the median age at the time of a CLL diagnosis, the median age ranges from 70 to 72 years[3]. The diagnosis of CLL in the majority of patients is done coincidentally when lymphocytosis is seen during a regular blood count. However, other patients may come forward with pain-free lymphadenopathy, or with symptoms which originate from cytopenia's or splenomegaly. Furthermore, patients can also come forward with classic B-symptoms, however, this only accounts for roughly 5%-10% of the patients[4]. CLL patients can be divided into two groups, the first group of patients needs therapy shortly after the diagnosis has been made and they have a clinical indication to start therapy according to approved international guidelines (iwCLL2018)[5]. The patients in the other group do not need therapy for maybe many years, these patients have indolent disease[1]. Another feature of CLL is that patients have an enhanced prevalence of developing secondary malignancies when they are compared to the widespread population, this was shown by several studies[6].

Secondary malignancies in CLL

The most common occurrence when it comes to progressive CLL, is the conversion of CLL to large B-cell non-Hodgkin's lymphoma (B-NHL), which is otherwise known as Richter's syndrome. However, also recorded in CLL patients, is the occurrence of secondary malignancies. For example the occurrence of secondary lymphoid malignancies, myeloproliferative disorders and the rise of solid tumors[7]. The most common secondary malignancy in CLL patients is skin cancer (Figure 1). A study by Ishdorj and colleagues showed a 2-fold increase in the incidence of cancers excluding the skin, and an 8-fold increase in the incidence of skin cancer in CLL patients[8]. However, there are also studies that showed an elevated risk of 2.3-3.1 for the rise of malignant melanoma after being diagnosed with CLL[9].

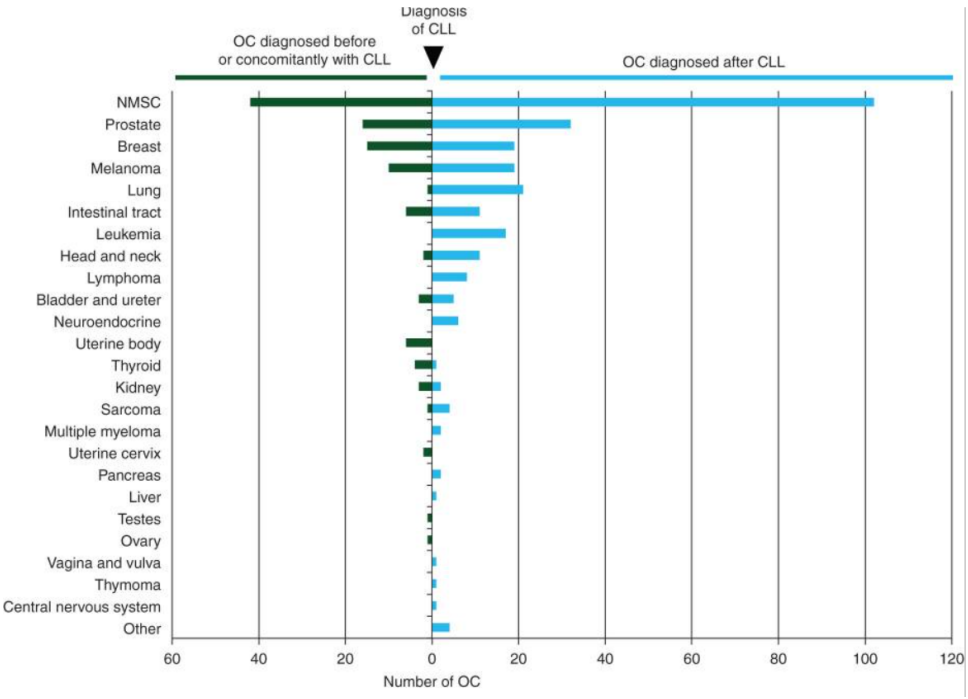

Figure 1: distribution of secondary malignancies by primary cancer type and time relationship with CLL diagnosis in all patients. OC = other cancer; NMSC = non melanoma skin cancer[6].

Additional studies have shown the same results when it comes to other malignant neoplasms, excluding skin cancer. These studies showed that secondary malignancies, happened 2.2 times more often in CLL patients compared to the widespread population[10]. In contrast to some cancers, such as melanoma, for which CLL patients have an increased risk to develop, there are also some cancers for which CLL patients have a decreased risk. These cancers include, among others, breast cancer, uterine cancer, gastrointestinal cancer and bladder cancer. It is hypothesized by Kumar and colleagues that this decreased risk is due to specific environmental and genetic factors that play a role in the development of these malignancies, which are independent of the development of CLL[6,11].

Results of multiple studies have shown that women have a relative lower risk than men to be struck with a secondary malignancy, which makes the male gender a risk factor for developing a secondary malignancy[10,11,12]. Other risk factors for developing a secondary malignancy are a lower platelet count, advanced age and ethnicity[6,11]. An often-debated issue is the role of systemic therapy associated with CLL, for example chemotherapy, in the development of a secondary malignancy. There is conflicting evidence regarding this issue, some studies suggest that there is no significant role for CLL therapy in the development of a secondary malignancy[6,10]. However, other studies suggest that there is a significant role for systemic CLL therapy in the development of a secondary malignancy. The cause of getting a secondary malignancy seems to be multifactorial, this includes shared genetic and environmental risk factors, a dysregulated immune system as well as a detection

bias. Since CLL patients have a higher frequency of going to the hospital for their controls, potential secondary malignancies will be detected sooner[11].

The development of a secondary malignancy in CLL influences the survival of CLL patients. A study by Falchi and colleagues showed that CLL patients who also have a secondary malignancy, have a median survival of 22.9 years. However, it is important to notice that in nearly half of the cases, the secondary malignancy was the cause of death. The same study showed that CLL patients without a secondary malignancy have a median survival of 16.2 years[6]. Another study by Kyasa and colleagues showed that in CLL patients with a secondary malignancy, in 34% of the cases, the secondary malignancy was the cause of death[13]. As mentioned previously, the cause of developing a secondary malignancy seems to be multifactorial. Therefore, it is important to understand the CLL pathogenesis, since this mechanism might play an important role in the development of a secondary malignancy.

CLL pathogenesis

In this paragraph, the main mechanism of CLL pathogenesis will be discussed, namely B-cell receptor (BCR) signaling and chronic antigen stimulation of the BCR, as well as the role of the immune system. It has been demonstrated, directly in patients with CLL, that the fundamental areas for CLL cell proliferation, are the secondary lymphoid organs. CLL cells form “pseudo follicles” in the lymph nodes, which are typical proliferation centers that copy normal B-cell follicles. In these areas, the CLL cells participate in molecular and cellular interactions, which mirror the molecular and cellular interactions of normal B-cells which develop in germinal centers in the course of adaptive immune responses. CLL cells are able to have interactions with T-cells and nurse-like cells, which gives rise to CLL cell activation. This CLL cell activation has a BCR and nuclear factor κB (NF-κB) gene signature. One of the gaps in our knowledge nowadays, about the CLL pathogenesis, is the fact that the exact mechanism that is fundamental for the activation of BCR signalling in CLL remains controversial[14].

As shown in Figure 2, normal BCR signaling occurs when a proper BCR is formed on the surface of the cell. This is the case when the antibody part of the BCR couples with the heterodimer of CD79A and CD79B. This complete BCR can bind an antigen (both microbial and autoantigens) and start signal transduction. This signaling is basically moderated by the single Immunoreceptor Tyrosine-based Activation Motif (ITAM) motifs, these motifs can be found in the cytoplasmic tail of CD79A and CD79B and each ITAM carries two tyrosine residues. Even when there is no antigen present, the BCR passes on low-level ‘tonic’ signals, this type of signaling is crucial for the survival of all mature B-cells. However, the presence of an antigen causes gathering of the BCR on the plasma membrane of the B-cell. This aggregation leads to the quick phosphorylation of the tyrosine residues

of the CD79A and CD79B ITAMs. This phosphorylation is done by SFKs, the phosphorylated ITAMs are then able to engage SYK kinase via its SH2 domain which can bind dually phosphorylated ITAMs, which will result in SYK activation. Then, this active BCR signalosome is able to engage a lot of adaptor proteins (BLNK and BTK) and additional kinases (PI3K). Eventually, antigen-stimulated BCR activation recruits several signaling cascades to modify differentiation, proliferation and survival of B-cells. For example, the calcium signaling, and subsequent activation of NF-κB is a pathway that is used by B-cells to conserve their viability[15].

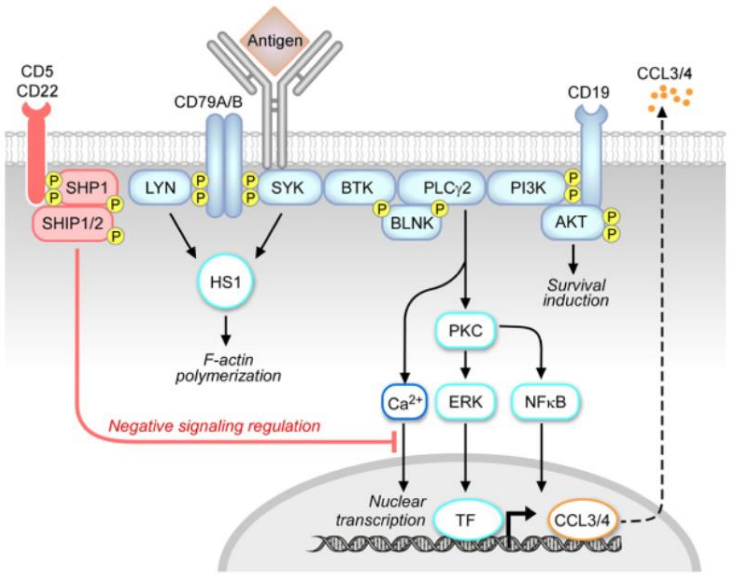

Figure 2: BCR signaling pathway[16].

Besides antigen-dependent signaling of the BCR, autonomous signalling can also take place. This happens when two CLL BCRs engage each other which also leads to BCR activation. In other words, the BCR can also be activated in the absence of antigens. Eventually, the activation of CLL BCRs, either antigen-dependent or antigen-independent, leads to the proliferation of CLL cells which is important in the pathogenesis of CLL[14,16]. There is clear evidence that the BCR is crucial for survival and proliferation of malignant B-cells. This evidence is derived from studies which show that there is an essential superior signaling activity of the BCR in CLL, this type of signaling is called chronic active BCR signaling, which leads to the essential activation of the NF-κB and PI3K pathways[17,18]. Chronic signaling of the BCR can be a consequence of auto-aggregation of BCRs. Auto-aggregation of BCRs occurs when the CLL-derived immunoglobulin variable domain sequences bind to the self-epitopes in the variable domains of other BCRs that are close by[18]. So, as explained previously BCR signaling plays a crucial role in CLL pathogenesis, eventually leading to tumor progression.

Elaborating on the role of the immune system in the pathogenesis of CLL, in the CLL microenvironment, there is an important role for the interaction of B-cells with T-cells. Moreover, the dynamics of the T-cell subsets have changed in CLL patients. Studies have shown that CLL

patients who have not received any treatment, have higher T-cell numbers and that these T-cells are oligoclonal, which is probably due to chronic antigen stimulation[17]. Other studies have shown that these higher T-cell numbers are due to a higher number of the $CD4^+$ T-helper cell subset, and that there is mostly an enhancement of effector and memory $CD4^+$ T-cells. But, there also seems to be a loss of naïve $CD4^+$ T-cells. Moreover, the $CD4^+$ T-cells of healthy individuals are thought to be less activated, compared to the $CD4^+$ T-cells of CLL patients. Nevertheless, studies have also shown that the T-cells of CLL patients have a higher expression of multiple inhibitory receptors and that there is a loss in the functional capacity of the T-cells, which results in an exhaustion phenotype. Besides the elevated numbers of $CD4^+$ T-cells, there are also elevated numbers of regulatory T-cells in CLL patients, which probably dampens the immune response. For the last subset of T-cells, the $CD8^+$ T-cells, studies have shown that it is most likely that the $CD8^+$ T-cells are able to recognize tumor specific antigens. However, despite this ability to recognize the antigens, the $CD8^+$ T-cells fail to respond and control disease, probably due to their functional exhaustion. A common feature of $CD4^+$ and $CD8^+$ T-cells in CLL is an effect that seems to be caused by the malignant CLL cells, namely the incapacity to form working synapses with CLL cells[19]. Next to these effects of CLL on the T-cells, the CLL lymphocytes are also able to kill plasma cells during cell-cell contact[20]. However, whether these effects also have an influence on the development of a secondary malignancy in CLL patients is not known.

Genetic abnormalities and treatments in CLL

Besides the immune dysregulation in CLL, it has been widely confirmed that CLL maintains a high level of genetic mutability[3]. This high level of genetic mutability might also play a role in the development of secondary malignancies. More than 80% of the CLL patients show chromosomal abnormalities, the most common chromosomal abnormalities are deletions on 6q (del(6q)), del(11q), del(13q), del(17p) and trisomy 12[21]. Besides these chromosomal abnormalities, there can also be mutations in the IGHV genes. In 60% of the cases, IGHV genes are mutated and in 40% of the cases, IGHV genes are unmutated[22]. Some of these chromosomal abnormalities have demonstrated prognostic value, which is also the case for the IGHV mutational status. For example, del(17p), a deletion in this location includes most of the time band 17p13, and this is the band with the important tumor suppressor gene TP53. Next to 17p deletions, 4% to 37% of the CLL patients have a mutated form of TP53. Patients with a 17p deletion including deletion of the gene TP53, or patients with a TP53 mutation, have an inferior prognosis if they are treated with chemo-immunotherapy. The poor prognosis is due to the fact that these patients do not respond to genotoxic

chemotherapies, this resistance cannot be repressed by adding anti-CD20 antibodies, in other words, by treating the patients with chemo-immunotherapy[2]. Even though these patients do not respond to chemo-immunotherapy, the rise of chemo-immunotherapy has played an enormous role in the management of CLL[23]. Due to chemo-immunotherapy, there has been a rise from 67.5% to 87.9% in the 5-year survival of CLL patients[24]. With the use of chemo-immunotherapy, the majority of CLL patients face long-term survival and reach complete remission[6]. Patients who have 17p deletions or TP53 mutations have high-risk CLL, these patients have shown to respond to BCR-targeted treatment, which improves their outcome[25]. In the past few years, some new therapeutic agents have been accepted by the Food and Drug Administration (FDA), which has resulted in an adaptation of the way that CLL is managed. Nowadays, the focus is rather on newer targeted therapy, than standard chemo-immunotherapy[11]. Consequently, due to all these treatment advances, people live for quite some time with their disease which means that they are vulnerable for complications. These complications include, for example, the occurrence of secondary malignancies.

Due to the association between secondary malignancies and CLL, the aim of this study is to elaborate a hypothesis to explain biologically the association between CLL and secondary malignancies using clinical data from CLL patients with secondary malignancies at the University Medical Centre Groningen (UMCG). In this study, there will be focused on four hypotheses:

1. One of the most important factors in the development of a secondary malignancy in CLL patients is the immune dysregulation.
2. One of the most important factors in the development of a secondary malignancy in CLL patients is the chronic signaling of the BCR.
3. One of the most important factors in the development of a secondary malignancy in CLL patients is the genetic abnormalities.
4. One of the most important factors in the development of a secondary malignancy in CLL patients is the treatment with systemic therapy.

MATERIALS & METHODS

After reviewing the patient database at the UMCG (epic), patients were selected based on being diagnosed with both, CLL and another malignancy or other malignancies. Variables related to the patient, disease and other malignancies were collected:

The following information about the patients was collected: birth date, age at time of the study, sex, presence of co-morbidities, date of last contact with the UMCG, enrollment in Oncolifes (a databank with data and body material of cancer patients) and, if applicable, date of death and cause of death.

The following information about the CLL was collected: date of diagnosis, age at time of CLL diagnosis, disease stage (both Rai stage and Binet stage), genetic aberrations, IGHV mutational status, karyotype, FISH, current status of CLL and if applicable, date and type of treatment.

The following information about the secondary malignancy of CLL patients was collected: date of diagnosis, histologic type of secondary malignancy, genetic aberrations and current state of the malignancy.

The data was organized and therefore, the secondary malignancies were divided in three categories: skin malignancies, solid tumors and hematological malignancies. To look at the influence of treatment on the development of a secondary malignancy, the date of CLL diagnosis was compared to the date of diagnosis of the secondary malignancy, then, these dates were also compared to the date of first treatment with systemic therapy for CLL, if this was applicable to the patient.
In order to compare the data from this study about CLL patients with a secondary malignancy, to data from previous study with CLL patients without a secondary malignancy, PubMed was searched. Using search terms as ‘’genetics in CLL patients” and “genetics AND CLL” papers were selected to compare the results from this study to results from previous studies. Using the same method, papers were also selected in order to compare the data from this study about the influence of treatment, to the data of other studies. Hence, search terms as “treatment AND CLL AND secondary malignancies” were used.

RESULTS

Data was collected from 84 patients with CLL and other cancer(s). From these 84 patients, 22 were female (26,2%) and 21 had died at the time of analysis (25%).

The patients were divided into two groups, patients ≤65 and patients >65 at the time of CLL diagnosis. Forty-two patients (50%) were 65 years or younger at the time of CLL diagnosis and 42 patients (50%) were older than 65 years at the time of CLL diagnosis. The mean age of the patients ≤65 was 55,1 years and the mean age of patients >65 was 77,1 years.

All of the included patients had at least one other malignancy. Thirty-two patients also had a third malignancy (38,1%), and 8 patients had a fourth malignancy (9,5%) (Table 1).

Table 1: Overview of the patient characteristics.

Patient characteristics	n (%)
Total number of patients	84
Female	22 (26,2%)
Dead	21 (25%)
Group 1: age ≤ 65	42 (50%)
Mean age group 1	55,1
Group 2: age > 65	42 (50%)
Mean age group 2	77,1
Third malignancy	32 (38,1%)
Fourth malignancy	8 (9,5%)
Secondary malignancy after treatment	25 (29,8%)

Fifty-six of the 84 patients (66,7%) developed a secondary malignancy without having received any treatment at all. Twenty-five of 84 patients (29,8%) developed a secondary malignancy after having received treatment (Table 1) (Figure 3). For the other 3 patients there was not enough data to comment on this.

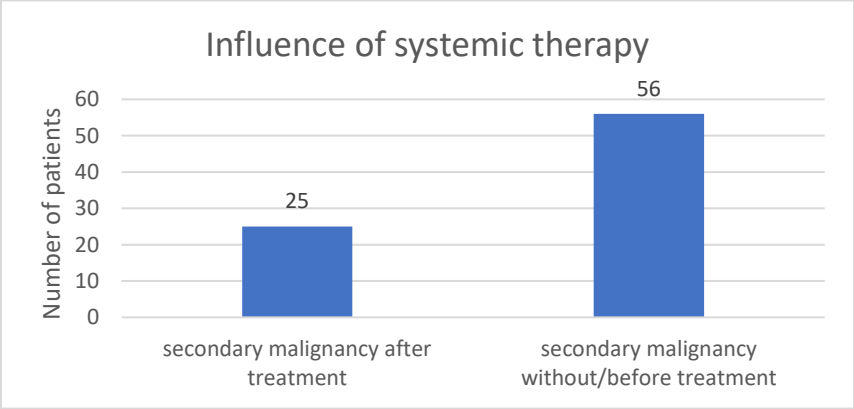

Figure 3: Influence of systemic therapy on the development of a secondary malignancy in CLL patients. N=84 patients, from 3 patients there was no data available.

The secondary, third and fourth malignancies were divided into three groups, based on the type of malignancy: skin malignancies, solid tumors and hematological cancers. From the secondary malignancies, 42 patients had a skin malignancy (50%), 35 patients had a solid tumor (41,7%) and 7 patients had another hematological malignancy (8,3%).

From the 25 patients that developed a secondary malignancy after they had received CLL treatment (Table 1), 13 developed a skin malignancy (52%), 10 developed another solid tumor (40%) and 2 developed another hematological malignancy (8%).

Analyzing the influence of genetic aberrations in CLL on the development of a secondary malignancy. Data showed that from all 84 patients with a secondary malignancy, 49 carried genetic aberrations (58,3%) in their CLL cells. In 8 patients no genetic aberrations were found (9,5%) and for the other 27 patients, no data was available.

To elaborate on the influence of the genetic aberrations in CLL cells on the development of a secondary malignancy, there was looked at which genetic aberrations occurred in CLL and the type of secondary malignancy that the patient had. Data showed that 23 of the 42 patients with a skin malignancy as secondary malignancy carried genetic aberrations in their CLL cells. This was also the case for 22 of the 35 patients with another solid tumor as secondary malignancy and for 4 of the 7 patients with a hematological malignancy as secondary malignancy. Data showed that del(13q) and trisomy 12 were the most common genetic aberrations in CLL patients with a secondary malignancy, this was seen 30 and 13 times respectively (Table 2) (Figure 4).

Table 2: Overview of the information available about the secondary malignancy in CLL patients. Information about the type of secondary malignancy, the treatment requirements of CLL and the presence of genetic aberrations in CLL.

				Genetic aberrations in CLL cells			
Secondary malignancy	Number of patients (n=84)	Diagnosed after CLL treatment (n=25)	Number of patients with genetic aberrations (n=49)	del(13q)	trisomy 12	del(11q) ATM	del(17p) TP53
Skin malignancy	42 (50%)	13 (52%)	23 (46,9%)	15	4	5	2
Solid tumor	35 (41,7%)	10 (40%)	22 (44,9%)	12	7	5	3
Hematological malignancy	7 (8,3%)	2 (8%)	4 (8,2%)	3	2	0	2
				30 (35,7%)	13 (15,5%)	10 (11,9%)	7 (8,3%)

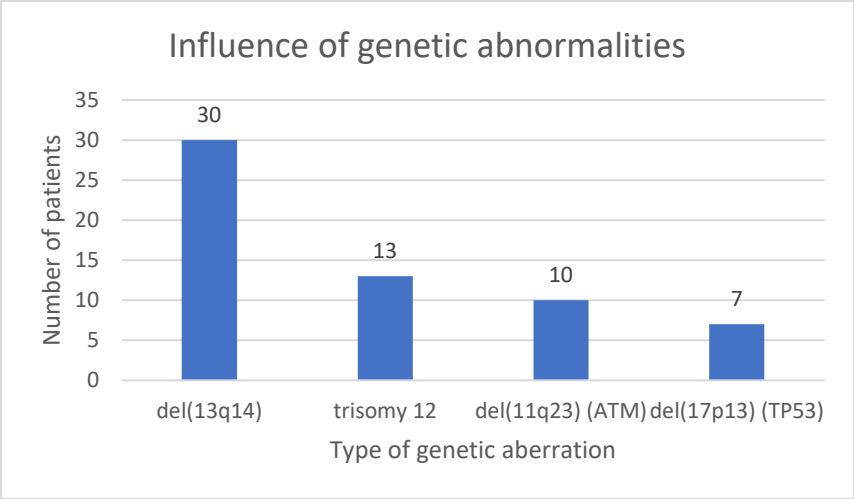

Figure 4: Influence of genetic abnormalities on the development of a secondary malignancy in CLL patients. N=49 patients with genetic aberrations.

As mentioned previously, there were 32 patients with a third malignancy. From these patients, 18 patients had a skin malignancy (56,3%), 13 patients had a solid tumor (40,6%) and 1 patient had another hematological malignancy (3,1%). Moreover, 13 patients from the 25 patients who developed a secondary malignancy after having received treatment (Table 1), also developed a third malignancy. From this group of patients, 10 developed a skin malignancy (76,9%), 2 another solid tumor (15,4%) and 1 another hematological malignancy (7,7%) (Table 3) (Figure 5).

Table 3: Overview of the information available about the third malignancy in CLL patients. Information about the type of third malignancy, the treatment requirements of CLL and the presence of genetic aberrations in CLL.

				Genetic aberrations in CLL cells			
Third malignancy	Number of patients (n=32)	Diagnosed after CLL treatment (n=13)	Number of patients with genetic aberrations (n=19)	del(13q)	trisomy 12	del(11q) ATM	del(17p) TP53
Skin malignancy	18 (56,3%)	10 (76,9%)	11 (57,9%)	3	3	3	0
Solid tumor	13 (40,6%)	2 (15,4%)	7 (36,8%)	4	1	0	0
Hematological malignancy	1 (3,1)	1 (7,7%)	1 (5,3%)	1	0	0	0
				8 (25%)	4 (12,5%)	3 (9,4%)	

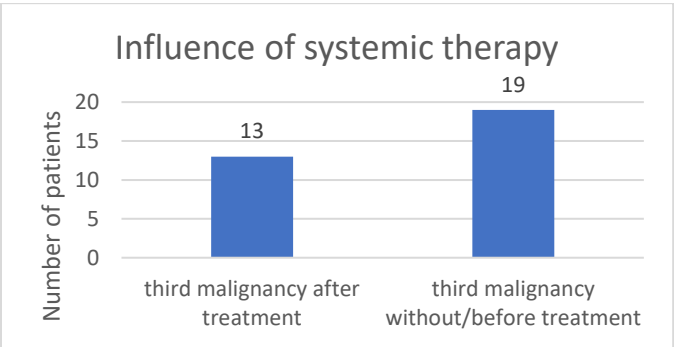

Figure 5: Influence of systemic therapy on the development of a secondary malignancy in CLL patients. N=32 patients with a third malignancy

Genetic aberrations were present in 19 patients with a third malignancy (59,4%), 5 patients did not carry any genetic aberrations and from 8 patients there was not enough data available. Data showed that 11 of the 18 patients with a skin malignancy as third malignancy carried genetic aberrations in their CLL cells. This was also the case for 7 of the 13 patients with another solid tumor as third malignancy and for the 1 patient with a hematological malignancy as third malignancy. In the group of patients with a third malignancy, del(13q) was still the most common genetic aberration, followed by trisomy 12, seen 8 and 4 times respectively (Table 3) (Figure 6).

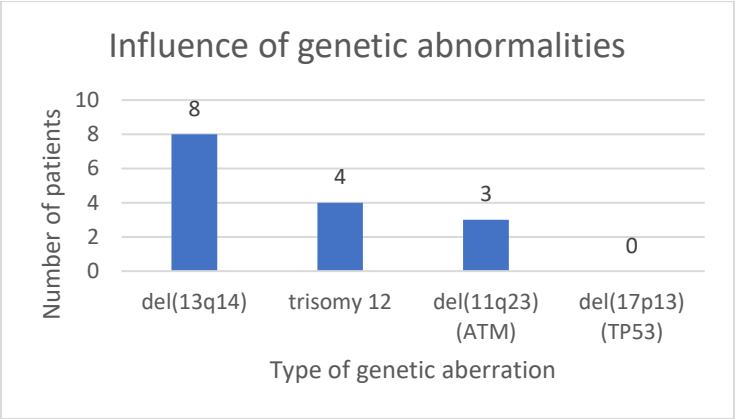

Figure 6: Influence of genetic abnormalities on the development of a secondary malignancy in CLL patients. N= 19 patients with genetic aberrations (not all patients carried one of the most common genetic aberrations).

As mentioned previously, there were 8 patients with a fourth malignancy. From these patients, 5 patients had a skin malignancy (62,5%), 2 patients had a solid tumor (25%) and 1 patient had another hematological malignancy (12,5%). The data showed that 4 patients, of the 25 patients who developed a secondary malignancy after having received treatment (Table 1), also developed a fourth malignancy and these were all skin malignancies. From the 8 patients with a fourth malignancy, 4 carried genetic aberrations in their CLL cells, 3 did not and from 1 patient there was not enough data. Interestingly, none of the patients with a fourth malignancy carried the genetic aberration of del(13q) which was the most common genetic aberration in patients with a secondary and third malignancy (Table 4).

Table 4: Overview of the information available about the fourth malignancy in CLL patients. Information about the type of fourth malignancy, the treatment requirements of CLL and the presence of genetic aberrations in CLL.

Fourth malignancy	Number of patients (n=8)	Diagnosed after CLL treatment (n=4)	Number of patients with genetic aberrations (n=4)
Skin malignancy	5 (62,5)	4 (100%)	3 (75%)
Solid tumor	2 (25%)	0	0 (0%)
Hematological malignancy	1 (12,5%)	0	1 (25%)

DISCUSSION

The aim of this study was to elaborate a hypothesis about the mechanism(s) to develop secondary malignancies in CLL patients from the analysis of CLL patient data from the UMCG register. Using the data that was collected after reviewing the patient database at the UMCG, our results confirm what has already been found in the literature. It was confirmed that males have a higher tendency of developing secondary malignancies than women, since only 26,2% of the patients was female[10]. It was also confirmed that younger patients with CLL are at a higher risk of developing a secondary malignancy since 50% of the patients was younger than 65 years[12]. So, this data suggests that a younger age at the time of CLL diagnosis, as well as the male gender are risk factors for developing a secondary malignancy. However, there are also studies that suggest that an advanced age at the time of CLL diagnosis is a risk factor for developing a secondary malignancy[6,11].

One hypothesis in this study stated that one of the most important factors in the development of a secondary malignancy in CLL patients is the occurrence of genetic abnormalities. The most common genetic aberrations in CLL patients without a secondary malignancy include del(13q14), trisomy 12, del(11q23) (ATM) and del(17p13), which is the same as results of this study show in CLL patients with a secondary malignancy. Moreover, in this study, there were only 10 patients, from the 49 who carried genetic aberrations, that did not carry either del(13q14) or trisomy 12. This is probably a result of del(13q14) and trisomy 12 being seen as early driver mutations in the development of CLL. Based on the results of this study, it would be likely that genetic aberrations play an important role in the development of a secondary malignancy, because genetic aberrations occur in more than half of the patients. However, comparing these results to the occurrence of genetic aberrations in CLL patients without a secondary malignancy showed that there is not much of a difference. A study by Puiggros et al. showed that del(13q14) is the most common genetic abnormality in CLL patients with an occurrence of >50%, this study showed an occurrence of 35,7%. It was also shown by Puiggros et al. that trisomy 12, del(11q23) (ATM) and del(17p13) occurred in 10-20%, 5-20% and 3-8% of the cases respectively. This study showed that trisomy 12, del(11q23) (ATM) and del(17p13) occurred in 15,5%, 11,9% and 8,3% of the cases respectively. So, the results from this study correspond to the results of the study by Puiggros et al.[21].

So, from these results there can be concluded that the genetic abnormalities in CLL patients with a secondary malignancy do not differ from the genetic abnormalities in CLL patients without a secondary malignancy, moreover, in the case of del(13q14) the genetic abnormality is even less common. It is not likely that a mechanism caused by the genetic aberrations plays a pivotal role in

the development of the secondary malignancy. Therefore, there can be concluded that the occurrence of genetic abnormalities, probably is not enough in the development of the secondary malignancy in CLL patients. Unfortunately, a limitation of this study was the fact that for 32,1% of the patients there was no data available about genetic abnormalities in their CLL cells. So, in order to be sure, future research in larger patient cohorts is needed.

Another hypothesis in this study stated that one of the most important factors in developing a secondary malignancy in CLL patients was the use of systemic therapy as treatment. Results showed that 29,8% of the patients developed a secondary malignancy after having received treatment, in the form of systemic therapy, for their CLL. However, 66,7% of the patients developed a secondary malignancy without having received any treatment. As explained previously, earlier studies generated controversial results about the influence of treatment on the development of secondary malignancies in CLL patients. A study by Tsimberidou et al. showed that 14,8% of the CLL patients with a secondary malignancy developed this secondary malignancy after having received treatment[10], which is less than the results from this study show. However, another study by Benjamini et al. showed that 28% of the CLL patients with a secondary malignancy developed this secondary malignancy after having received treatment[26], which is in concordance with the results from this study. Nevertheless, from these results there can be concluded that treatment in the form of systemic therapy is probably not one of the most important factors in the development of a secondary malignancy in CLL patients. In order to be sure, future research should focus on a larger patient population and distinguish between several types of systemic therapies.

So, since genetic abnormalities and systemic therapy do not seem to have a pivotal role in the development of secondary malignancies in our cohort. Next, there will be focused on the other two hypotheses, immune dysregulation and chronic stimulation of the BCR. Unfortunately, due to the covid-19 pandemic, the means to test these hypotheses were not available. Therefore, an experimental set-up for future research in order to test these hypotheses will be given.

FUTURE RESEARCH

Hypothesis 1: “One of the most important factors in the development of a secondary malignancy in CLL patients is immune dysregulation”

To test the first hypothesis, the following questions should be answered.

- What are the differences in immune dysregulation between CLL patients with and without a secondary malignancy?
- Are there specific markers of the immune system (numbers and/or function) at the time of CLL diagnosis, which could be predictors for developing a secondary malignancy in CLL patients?
- If there are specific markers, then could the flow panel be expanded at the time of diagnosis to predict the patients that are at risk for developing a secondary malignancy?

In order to answer these questions, the following experiments could be performed:

The data that will be generated from the following experiments, from both CLL patients with and without secondary malignancies should be compared each other.

Draxler et al. presented a new protocol in two 15-color antibody panels for flow cytometry. Both, information regarding the phenotype and the functionality of the identified subsets (innate immune cells and T-cell subsets) can be obtained. There was stated that the information for each cell population covers maturation, activation, migration, responsiveness to cytokine signaling, as well as direct cellular interactions. The protocol was optimized for RBC-lysed human whole blood as well as for peripheral blood mononuclear cells (PBMCs)[27]. So, in order to study the differences in immune dysregulation between CLL patients with and without a secondary malignancy, blood samples have to be collected from these patients. Then, the protocol as described by Draxler et al. has to be followed which will result in samples that can be used for immunophenotyping. Using immunophenotyping, both myeloid and lymphoid cell population will be studied in order to map the innate immune response as well as the T-cell response.

For phenotypic identification of the myeloid cell populations, there will be made use of SSC, as well as the markers CD33, CD123, CD14, CD16, HLA-DR, CD11c, CD141, and CD1c. Then, for the evaluation of cell activation, migration, function, and cell viability, there will be made use of additional markers. These markers are CD83, CD86, CCR4, CCR7, PD-L1, TNFR2, and Zombie Yellow for live/dead discrimination (Table 5).

Table 5: Phenotyping (A) and functional assessment (B) of myeloid cell populations using SSC and surface markers in the myeloid cell population panel[27].

A: MYELOID CELL POPULATIONS								
CD141+ cDC	SSC low	CD33+	CD123−	CD14−	CD16−	HLA-DR+	CD11c+	CD141+
CD1c+ cDC	SSC low	CD33+	CD123−	CD14−	CD16−	HLA-DR+	CD11c+	CD1c+
pDC	SSC low	CD33+	CD123+	CD14−		HLA-DR+	CD11c−	
Classical monocytes	SSC med	CD33+	CD123−	CD14+	CD16−	HLA-DR+		
Intermediate monocytes	SSC med	CD33+	CD123−	CD14+	CD16+	HLA-DR+		
Non-classical monocytes	SSC med	CD33+	CD123−	CD14−	CD16+	HLA-DR+		
MO-MDSC	SSC med	CD33+	CD123−	CD14+	CD16+	HLA-DR−		

B: MYELOID CELLS: FUNCTIONAL MARKERS			
Cell activation/maturation	CD83	CD86	HLA-DR
Cell migration	CCR4	CCR7	
Inhibitory signaling	PD-L1		
Responsiveness to signaling	TNFR2		
Cell viability	Zombie yellow for staining intracellular amines		

For phenotypic identification of the lymphoid cell populations, there will be made use of SSC, as well as the markers CD3, CD4, CD8, CD56, CD25, and FoxP3. Then, for the evaluation of cell activation, migration, function, and cell viability, there will be made use of additional markers. These markers are CD45R0, CCR4, CCR7, PD-1, CD95, TNFR2, LAP, CTLA4, and Zombie Yellow for live/dead discrimination (Table 6)[27].

Table 6: Phenotyping (A) and functional assessment (B) of lymphoid cell populations using SSC and surface markers in the lymphoid cell population panel[27].

A: LYMPHOID CELL POPULATIONS						
CD4+ T cells	SSC low	CD3+	CD56−	CD4+		
Treg	SSC low	CD3+	CD56−	CD4+	CD25+	FoxP3+
CD8+ T cells	SSC low	CD3+	CD56−	CD8+		
CD8+ Teff	SSC low	CD3+	CD56−	CD8+	CD45R0+	CCR7−
CD8+ Tmem	SSC low	CD3+	CD56−	CD8+	CD45R0+	CCR7+
NK cells	SSC low	CD3−	CD56+			
NKT cells	SSC low	CD3+	CD56+			

B: LYMPHOID CELLS: FUNCTIONAL MARKERS		
Cell activation/maturation	CD45R0	CCR7
Cell migration	CCR4	CCR7
Programmed cell death	PD-1	CD95
Responsiveness to signaling	CTLA4	TNFR2
TGF-β release	LAP	
Cell viability	Zombie yellow for staining intracellular amines	

Even though the fact that flow cytometry is a very functional and high-throughput technique, flow cytometry is restricted by the number of antigens that can be determined at the same time due to the spectral overlap of fluorophores. Another technique that can be used to test the first hypothesis is single-cell RNA sequencing (scRNAseq)[28].

Single-cell analysis has one requirement, namely the creation of a single-cell suspension. However, this process is relatively easy for CLL due to the fact that suspension cells can easily be acquired from the blood and bone marrow via venipuncture and bone marrow biopsy respectively. Moreover, CLL also frequently occurs in the lymph nodes, these can be sampled by performing a biopsy. Then, by using standard tissue disaggregation techniques, single cells can be acquired. The single-cell techniques that are available nowadays have a range of qualities which makes a number of the techniques more applicable for discovery, and other techniques more applicable for validation. Some of the techniques that are suited for discovery are whole-genome sequencing (WGS), whole-exome sequencing (WES) and whole transcriptome sequencing (RNA-seq).

The use of these single-cell analysis techniques has already provided us with a lot of new information about CLL. For example, using WGS, Zhao et al. discovered somatic copy number alterations (sCNA) that were associated with CLL. However, opposite to single-cell WGS and/or WES, there are also multiple studies that have assessed the co-expression of mutated events related to CLL using targeted assays relevant for single CLL cells. This was done in order to make more finished and precise phylogenetic trees. Using this knowledge, CLL subclones were identified. These subclones did not have explicit driver mutations and the effect of new mutations on cancer-driver functions was confirmed.

It was proposed by multiple studies that there is a need for a single-cell approach in order to identify transcriptionally defined subpopulations. Using single-cell approaches, it is possible to examine the transcriptional signature of CLL cells covering more than one time point. A study discovered variations in biological processes, including immune responses and cell cycle processes, of single CLL cells. These variations validated matched DNA mutational signatures and also discovered the deregulation of genes that are important for phospholipid binding and protein folding, which are sides of the CLL biology that were not acknowledged earlier on. In contrast, it has long been acknowledged that CLL cells are in close contact with the microenvironment. Due to the heterogeneity of the immune cell populations, there are benefits for single-cell analysis over the regular bulk approaches. For example, insights that have to come from single-cell analysis have the power to clarify the reasons of the inferior responses of CLL patients to checkpoint inhibitors and CAR T-cell therapy. In order to develop new therapies, or to improve existing therapies, there is a need for more knowledge about the components which regulate the immune system. This is knowledge that can be gained using single-cell approaches[28].

In addition, scRNAseq has already been used in order to explain the fundamental regulatory dynamics of successful treatment with ibrutinib, a Bruton tyrosine kinase (BTK) inhibitor. This showed that the effect of ibrutinib relies on a consistent regulatory program. However, results also showed that there is variation between patients in the speed of execution of this regulatory program[29]. Moreover, the transcriptional heterogeneity of CLL cells was discovered using scRNAseq (Figure 7)[30].

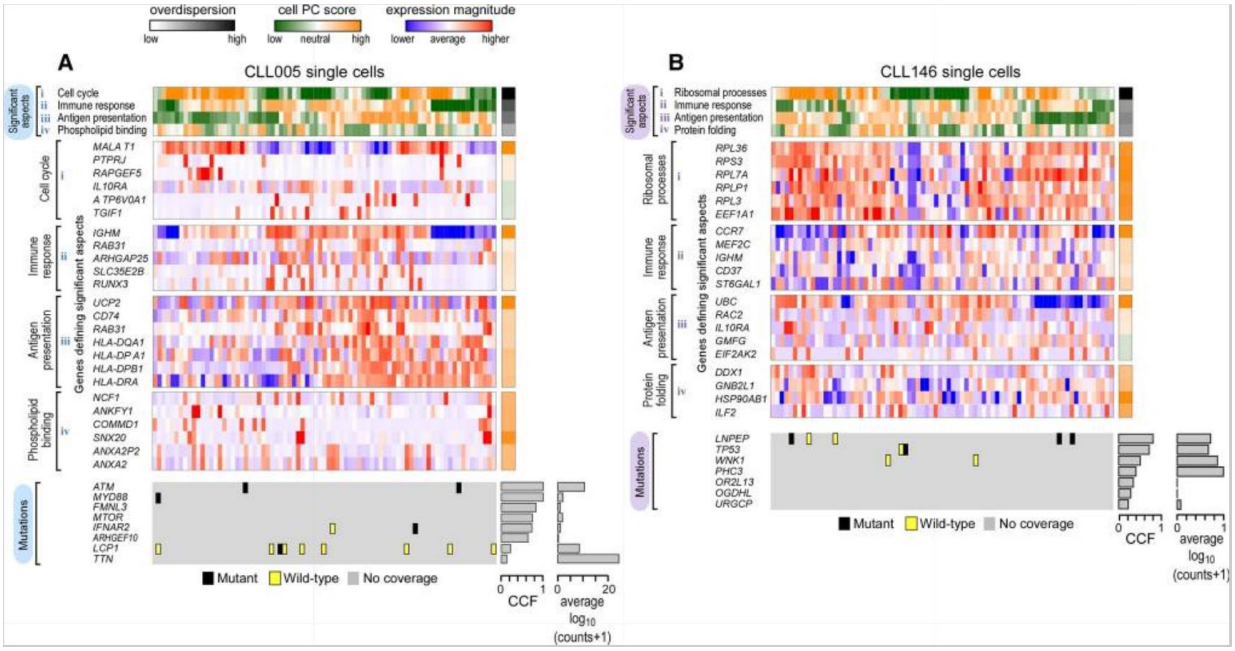

Figure 7: CLL transcriptional heterogeneity revealed by scRNAseq[30].

So, in order to systemically analyze the differences in gene expression between CLL patients with and without a secondary malignancy, scRNAseq can be performed. This can be done using the total PBMC population of the patients in order to analyze both, the non-malignant immune cells as well as the transcriptomes of CLL cells. Protocols for droplet-based scRNAseq, preprocessing and analysis of scRNAseq data can be found in a study performed by Rendeiro et al.[29].

Results might show that there is a significant difference between the immune dysregulation of CLL patients with and without secondary malignancies. This immune dysregulation can for example be seen in the number of cells that are present, or in the (altered) function of the immune cells. When these results are observed, then there should be tested whether this is also the case at the time of CLL diagnosis. When the same problems with immune dysregulation are seen at the time of CLL diagnosis, there can be stated that this factor, the amount of immune dysregulation, can be a predictor for developing a secondary malignancy in CLL patients. So, then there should be paid attention to this factor as well when diagnosing someone with CLL. In order to accomplish this, the CLL flow panel which is very limited at the moment and which is used to diagnose someone with CLL,

should be expanded with the markers that might come forward in this study. This way, in the future, it might be possible to predict whether patients are vulnerable for developing a secondary malignancy and it might be possible to prevent this secondary malignancy from happening.

Hypothesis 2: “One of the most important factors in the development of a secondary malignancy in CLL patients is chronic signaling of the BCR”

To test the second hypothesis, the following question should be answered.

- What is the difference in BCR signaling between CLL patients with and without a secondary malignancy?

In order to answer this question, the following experiments could be performed:

The data that will be generated from the following experiments, from both CLL patients with and without secondary malignancies should be compared each other.

In order to test the hypothesis, there can be studied whether the amount of chronic BCR signaling is the same in CLL patients with a secondary malignancy as in CLL patients without a secondary malignancy. This can be done by looking at the amount of proliferation of the cells, since proliferation is one of the results from BCR signaling. In order to look at the amount of proliferation, there can be made use of a Carboxyfluorescein succinimidyl ester (CFSE)-based proliferation assay[31,32](ThermoFisher). In order to perform the CFSE-based proliferation assay, the CLL cells should be stained with CFSE first. Then, the same protocol that was also used by López-Guerra et al. and Mongini et al. will be followed. The CFSE-labeled CLL cells have to be cultured in an enriched medium with added insulin/transferrin/selenium supplement. The enriched medium alone is normally used for normal B-cell replication in long-term cultures. Important to note is that the medium that will be used, including the added supplement, carries 2-ME, a reducing agent. This agent replaces one of the functions that is normally a function of bone marrow stromal cells, namely the converting of cystine to cysteine. This conversion is needed by the CLL cells for the uptake and use of this amino acid in the glutathione synthesis for retained viability. In 96-well plates, the cultures will be frequently established at 10^5 cells per 200µL volume with triplicates for each culture condition. Then, recombinant human IL-15 and CpG DNA TLR-9 ligand will be added at a final culture concentration of 15ng/mL and 0,2µM, respectively. Finally, after 6 days of incubation, the cells should be collected and stained with CD19-PE and Annexin-V-Pacific Blue. The CLL proliferation can be measured using an Attune acoustic cytometer, based on the median fluorescence intensity on the $CD19^+$ and

Annexin-V-negative cell population, the proliferation can be measured as the reduction of CFSE fluorescence. A more detailed protocol can be found in the studies performed by López-Guerra et al. and Mongini et al.[31,33].

Besides measuring the amount of proliferation, the activity of both PI3K and NF-κB can be measured. These variables should be measured in CLL cells of patients. The activity of PI3K can be measured using the PI3 Kinase Activity/Inhibitor ELISA Kit (Creative Diagnostics), or by measuring the activity of the Forkhead Box O (FOXO) transcription factor. The activity of the FOXO transcription factor is a reversed readout of the activity of the PI3K pathway. Using a knowledge-based Bayesian network computational model, the FOXO activity can be deduced from mRNA expression levels of 26 high evidence FOXO target genes. For the computational model, the mRNA levels can be used as input, these were acquired from Affymetrix expression microarray data. This technique of measuring the PI3K pathway activity works because FOXO is normally inactive in cells that are dividing, which is associated with an active PI3K pathway. This is for example the case in malignant tumors and in clonally expanding lymphocytes. FOXO becomes active when these cells are treated with drugs that are able to inhibit the PI3K pathway, for example when CLL patients are treated with idelalisib. A problem with using this method was the fact that the interpretation regarding the PI3K pathway activity was not always simple. This was due to the fact that in cancer tissue, cellular oxidative stress was also able to activate FOXO. And cellular oxidative stress can be a consequence of an active PI3K pathway which causes metabolic changes and quick cell division, which leads to high levels of reactive oxygen species (ROS). In order to distinguish this kind of FOXO activity, from normal FOXO activity, oxidative stress was defined as high FOXO activity in combination with higher levels of SOD2 mRNA (Figure 8)[34,35].

Figure 8: Distinction between tumor suppressive and oxidative stress mode of FOXO activity[35].

A detailed protocol for using this method in order to measure the PI3K activity of cells can be found in the studies performed by Van de Stolpe and van Ooijen et al.[34,35].

The activity of NF-κB can be measured using NF-κB (p50, p52, p65, c-Rel, RelB) Transcription Factor Assay Kit (abcam). In order to use this, cell lysate, nuclear extracts are needed. To prepare the nuclear extracts, the Nuclear Extraction Kit (ab113474) can be used. Moreover, the NF-κB DNA binding activity can also be determined using an electrophoretic mobility shift assay. The

electrophoretic mobility shift assay relies on the aspect that complexes which consist of DNA and protein migrate in a slower pace than DNA that is not bound to protein in native polyacrylamide. This results in a “shift” in the migration of the labeled DNA. A detailed protocol for this assay can be found in a study performed by Wang et al.[36].

When results show that there is an elevation in the amount of chronic BCR signaling in CLL patients with a secondary malignancy compared to CLL patients without a secondary malignancy, then it might be helpful to look at this aspect when patients are diagnosed with CLL. When the same results are observed at the time of CLL diagnosis, then in the future it might be possible to identify the CLL patients that are vulnerable for developing a secondary malignancy. Even when this is not the case at the time of CLL diagnosis, this information is helpful because it might be better for patients with a higher level of chronic BCR signaling to be treated with targeted therapies like BTK inhibitors earlier on.

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