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XXV Ciclo

Traditional and new diagnostic and prognostic markers in breast cancer

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CONTENTS

INTRODUCTION

Breast cancer is the most common malignant tumor in women around the world, comprising 25% of all cancer cases in women [1]. Because of its high morbidity, breast cancer seriously affects women's health and life quality. At present, surgical resection is the main treatment strategy for patients with breast cancer. Adjuvant chemotherapy, endocrine therapy, radiation therapy, targeted therapy and other comprehensive treatments have also significantly improved disease free survival and overall survival. However, postoperative recurrence and metastasis represent still important problems for both clinicians and patients. Thus, there is an urgent need to establish effective models for evaluating prognosis of breast cancer. The Nottingham Prognostic Index (NPI) established in 1982 is a widely used clinico-pathological score system for primary breast cancer prognostication [2]. The NPI combines nodal status, tumor size and histological grade in a simple formula. It stratifies breast cancer patients into three prognostic groups, good, moderate and poor [3].

Grading of breast cancer

The grading of a cancer in the breast depends on the microscopic similarity of breast cancer cells to the normal breast tissue, and classifies the cancer as well differentiated (low-grade), moderately differentiated (intermediate-grade), and poorly differentiated (high-grade), reflecting progressively less normal appearing cells that have a worsening prognosis. Although grading is fundamentally based on how biopsied, cultured cells behave, in practice the grading of a given cancer is derived by assessing the cellular appearance of a tumor. The closer the appearance of the cancer cells to normal cells, the slower their growth and the better the prognosis. If cells are not well differentiated, they will appear immature, will divide more rapidly, and will tend to spread, resulting in a worse prognosis for the patient. To well differentiated tumor cells is given a grade of 1,

moderate is grade 2, while poor or undifferentiated cells is given a higher grade of 3 or 4 (depending upon the scale used). [4]

The Nottingham (also called Elston-Ellis) modification [4] of the Scarff-Bloom-Richardson grading system [5-6] is recommended, which grades breast carcinomas by adding up scores for tubule formation, nuclear pleomorphism, and mitotic count, each of which is given 1 to 3 points [7].

Tubule formation

This parameter assesses what percent of the tumor forms normal duct structures. In cancer, there is a breakdown of the mechanisms that cells use to attach to each other and communicate with each other, to form tissues such as ducts, so the tissue structures become less orderly. The overall appearance of the tumor has to be considered and according to this criterion classification will be made as follows:

1 point: tubular formation in more than 75% of the tumor

- 2 points: tubular formation in 10 to 75% of the tumor
- 3 points: tubular formation in less than 10% of the tumor

Nuclear pleomorphism

This parameter considers the appearance of nuclei and assesses whether they appear uniform, like those in normal breast duct epithelial cells, or whether they are larger, darker, or irregular (pleomorphic). In cancer, the mechanisms that control genes and chromosomes in the nucleus break down, and irregular nuclei and pleomorphic changes are signs of abnormal cell reproduction. According to nuclear appearance tumor cells will be classified as follows:

- 1 point: nuclei with minimal variation in size and shape
- 2 points: nuclei with moderate variation in size and shape

- 3 points: nuclei with marked variation in size and shape

Mitotic count

This parameter assesses how many mitotic figures (dividing cells) can be seen in 10x high power microscope field. More specifically, mitotic figures are counted only at the periphery of the tumor, and counting should begin in the most mitotically active areas.

One of the hallmarks of cancer is that cells divide uncontrollably. The more dividing cells will be observed, the worse the cancer will behave. According to this parameter tumor cells will be classified as follows:

1 point: 0-9 mitotic counts per 10 fields under X25 objective using the Leitz Ortholux microscope, 0-5 mitotic counts per 10 fields under X40 objective using the Nikon Labophot microscope, or 0-11 mitotic counts per 10 fields under X40 objective using the Leitz Daiplan microscope.

2 points: 10-19 mitotic counts per 10 fields under X25 objective using the Leitz Ortholux microscope, 6-10 mitotic counts per 10 fields under X40 objective using the Nikon Labophot microscope, or 12-22 mitotic counts per 10 fields under X40 objective using the Leitz Daiplan microscope.

3 points: Over 19 mitotic counts per 10 fields under X25 objective using the Leitz Ortholux microscope, over 10 mitotic counts per 10 fields under X40 objective using the Nikon Labophot microscope, or over 22 mitotic counts per 10 fields under X40 objective using the Leitz Daiplan microscope.

The scores for each of these three criteria are added together to give a final overall score and a corresponding grade as follows:

- 3-5 **Grade 1** tumor (**well-differentiated**).

- 6-7 **Grade 2** tumor (**moderately differentiated**).

•8-9 **Grade 3** tumor (**poorly differentiated**).

Staging of breast cancer

Staging of breast cancer is one aspect of breast cancer classification that assists in making appropriate treatment choices, when considered along with other classification aspects such as Estrogen Receptor (ER) and Progesterone Receptor (PR) levels in the cancer tissue, index of proliferation (Ki67/Mib1) and the Human Epidermal growth factor Receptor 2 (HER2/neu) status.

Classical immunohistochemistry (IHC) markers such as ER, PR and HER2, together with traditional clinico-pathological variables including tumor size, tumor grade and nodal involvement, are conventionally used for patient prognosis and management [8-9]. The advent of high-throughput platforms for gene expression analysis has shown that tumor cell response to treatment is not determined by anatomical prognostic factors but rather intrinsic molecular characteristics that can be probed using molecular methods [11-12]. This conceptual change has led to a new paradigm on how breast cancer patients are stratified and treated, which provides an incremental increase of the reproducibility and accuracy of disease prognosis and therapeutic decision making [10]. Integrating information from multiple levels or dissecting this problem from the pathway point of view, has led to an expanding spectrum of breast cancer subtypes or the other way around. With our incremental knowledge on this complex tumorigenesis progress, novel molecules with emerging roles are gaining their importance which, despite contributing to deciphering breast cancer heterogeneity, complicate our understanding towards subtyping of this complex disease.

Gene expression profiling and intrinsic subtypes

With the development of microarrays, gene expression profiling (GEP) has been used for breast cancer prognosis, specifically aiming at identifying patients with sufficiently good prognosis to allow the safe omission of adjuvant chemotherapy [12-13]. The pioneer studies conducted by Sørlie et al. [16] reported a distinctive 'molecular portrait' of breast cancer using 456 cDNA clones, according to which tumors were classified into five intrinsic subtypes with distinct clinical outcomes, namely luminal A, luminal B, HER2 over-expressing, basal and normal-like tumors [14-15]. The rationale underlying such classification is that the differences underlying the gene expression patterns among cancer subtypes reflect the fundamental differences of the tumors at the molecular level [16]. Each of the five intrinsic subtypes is nicely mapped to an IHC-defined subtype (**Table 1**) except for the normal-like tumors which account for 7.8% of all breast cancer cases in a lymph-node negative cohort [17], share a similar IHC status with the luminal A subtype and are characterized by a normal breast tissue profiling [18]. These five intrinsic subtypes have been confirmed by several other studies with varying numbers of genes included in the signatures. For instance, Hu et al. identified a signature containing 306 genes that can distinguish these subtypes with significant differences observed on relapse-free and overall survival [19]. Parker et al. reported a 50-gene classifier (PAM50, which contains mostly hormone receptor and proliferation-related genes, and genes exhibiting myoepithelial and basal features), which has significant prognostic and predictive values on breast tumors [20-22], and can be widely applied in the clinical setting [23]. It is worth noting that although different in gene composition, the signatures identified by different studies should converge to the pathways they imply, based on which the same sample should not be classified into different categories. However, this is not the case due to, e.g., lack of stringent standardization of the methodology and breast cancer intrinsic subtype definition [24].

The development of tissue microarray (TMA) technology has enabled the validation of gene signatures at the translational level. A study exploring the combined protein expression profiles of a large panel of well-characterized commercially available biomarkers revealed 5 major groups [25]. In particular, this study identified two large subtypes exhibiting luminal epithelial cell phenotypic characteristics, hormone receptors positivity, absence of basal epithelial phenotypic features and HER2 protein over-expression; two subgroups characterized by high HER2 positivity and negative hormone receptor expression, which differ from each other by MUC1 and E-cadherin expression; and one group characteristic of strong basal epithelial marker expression, p*53* positivity, absent hormone receptor expression and weak luminal epithelial and cytokeratin expression [25]. These subtypes accord well with those intrinsic subtypes based on GEP, which confirms the biological heterogeneity of breast cancer and demonstrates the clinical relevance of the intrinsic subtypes identified using high-throughput technologies [25].

Though Sørlie's subtyping has set the standard for intrinsic breast tumor categorization, other classifications also exist. For instance, Sotiriou et al. identified 6 groups among breast carcinomas using a signature containing 706 cDNA probe elements, which include 3 luminal-like, 1 HER2-like and 2 basal-like subtypes [26]. Fan et al. have suggested a 70-gene signature to classify tumors into 4 groups where the normal-like subtype was not identified according to Sørlie's subtyping [27]. Lehmann et al. have subdivided triple negative tumors into 6 stable groups, two basal-like (BL1 and BL2), an immunomodulatory (IM), a mesenchymal (M), a mesenchymal stem-like (MSL) and a luminal androgen receptor (LAR) subtype, by using GEP. The BL1 subtype is heavily enriched in cell cycle and cell division genes, suggesting the potential response to anti-mitotic agents such as taxanes (paclitaxel or docetaxel) of tumors belonging to this subtype. The BL2 subtype displays unique gene ontologies involving growth factor signaling, and has features suggestive of a basal/ myoepithelial origin. The IM subtype is

enriched for genes involved in immune cell processes. The M subtype displays a variety of unique gene ontologies that are heavily enriched in cell motility, ECM receptor interaction, and cell differentiation pathways. The MSL subtype shares genes for similar biological processes with the M subtype. Besides, this subtype contains genes involved in growth factor signaling, and displays low expression of claudin 3, 4, 7 (similar to the claudin-low subtype). The LAR subtype is ER negative, but displays luminal gene expression patterns [28].

Table 1. Summary of the breast tumor molecular subtypes

Intrisic subtype	IHC status	Grade	Outcome
Luminal A	ER+ PR+ HER2- Ki67-	1-2	Good
Luminal B	ER+ PR+ HER2- Ki67+	2-3	Intermediate
	ER+ PR+ HER2+ Ki67+		Poor
HER2 over-expression	ER- PR- HER2+	2-3	Poor
Basal	ER- PR- HER2-	3	Poor
Normal-like	ER+ PR+ HER2- Ki67-	1-2-3	Intermediate

Expression and clinical features of basic intrinsic subtypes

Luminal tumors

The luminal-like tumors express hormone receptors, with expression profiles reminiscent of the luminal epithelial component of the breast [18]. These patterns include the expression of luminal cytokeratins 8/18, ER and genes associated with ER activation such as *LIV1* and *CCND1* [18, 26]. At least two subtypes exist within luminal-like tumors, i.e., luminal A and luminal B. Approximately speaking, the luminal A and luminal B tumors each represents the [ER+-PR-+/HER2- (tumors with ER or PR positivity and HER2 negativity) and [ER+-PR+-HER2+ (tumors with ER or PR positivity and HER2 positivity)

subtype, respectively, using the IHC nomenclature introduced in the previous section [30]. However, this equivalence does not always hold as, e.g., only part of luminal B tumors are HER2+ [9].

Luminal A tumors have higher expression of ER-related genes and lower expression of proliferative genes than luminal B cancers [14, 16].

Luminal B tumors tend to be of higher grade than luminal A tumors. Luminal tumors are the most common subtypes among breast cancer, with luminal A being the majority. In the Carolina Breast Cancer Study [31], luminal breast tumors represent 64.3% of all patients, where luminal A cancers account for 54.3% (i.e., 57%, 67%, 40% and 55% of pre-menopausal white, post-menopausal white, pre-menopausal African American and post-menopausal African American women, respectively). In general, the luminal subtypes carry a good prognosis, and luminal B tumors have a significantly worse prognosis than the luminal A subtype [16]. Luminal tumors respond well to hormone therapy but poorly to conventional chemotherapy [29]. Treatment response differs between luminal subtypes. According to the Recurrence Score, which is resulted from a RT-PCR based 16-gene predictor (half of them are ER and proliferation-related genes), tumors with low Recurrence Scores are luminal A while those with high Recurrence Scores are luminal B [32]. Thus, luminal A tumors could be adequately treated with endocrine therapy, while luminal B tumors, which are more proliferative, may benefit more from the combined therapeutic strategy of chemotherapy and hormonal treatment. Other targeted approaches such as anti-angiogenic strategies were suggested to be effective for luminal tumors as well. For example, the anti-VEGF antibody bevacizumab was shown to improve progression free survival in metastatic breast cancer when combined with paclitaxel, among which 60% of the patients carrying luminal tumors [29]. In 2012, the mTOR inhibitor everolimus (Afinitor) was approved in combination with exemestane for treating

ER-positive, HER2-negative advanced breast cancer that recurs on standard therapies [33]. In addition, Palbociclib (under development by Pfizer), a cyclin-dependent kinase (CDK) 4/6 inhibitor, is approaching approval for treating such patients on the basis of data from a phase II study [34].

HER2 over-expressing tumors

The intrinsic HER2 over-expression tumors refer to those identified using gene expression array, which is similar to the ER-PR-HER2+ (ER negative, PR negative, HER2 positive) subgroup by immunostaining or fluorescence *in situ* hybridization (FISH) [30]. However, tumors classified by these two systems do not perfectly match, as not all clinically HER2-positive tumors show changes at the transcriptional level. The HER2 over-expression tumors are characterized by over-expressing other genes in the HER2 amplicon such as *GRB7* [16, 33] and *PGAP3* [35]. 40% to 80% of these tumors harbor *TP53* mutation. HER2 over-expressing tumors are more likely to be of grade 3. No association with age or race was found for HER2 over-expressing tumors [27], as well as known risk factors [29, 36]. Though HER2 overexpressing breast tumors carry a poor prognosis [14, 16, 26], they are sensitive to anthracycline and taxane-based neoadjuvant chemotherapy, with significantly higher pathological complete response than luminal breast tumors [29]. The poor prognosis of this subtype as well as the basal tumors seem to derive from a higher risk of early relapse among those without a complete eradication of tumor cells, and cancers of these two classes are suggested to derive the most benefit from improvements in chemotherapy [29]. Unlike the basal tumors, molecularly targeted agents such as the anti-HER2 monoclonal antibody trastuzumab are available for HER2 over-expressing cancers. Not all HER2 over-expressing tumors respond to trastuzumab. *PTEN* loss [37] and *CXCR4* up-regulation [29] have been implicated in trastuzumab resistance, which provides targets in the combined strategies to improve clinical outcome in the future.

Basal tumors

As discussed before, the basal subtype is composed of ER-PR-HER2- (triple negative) tumors with expression profiles mimicking that of the basal epithelial cells of other parts of the body and normal breast myoepithelial cells [18]. Such expression patterns include lack or low expression of hormone receptors and HER2, and high expression of basal markers (such as keratins 5, 6, 14, 17, EGFR) and proliferation related genes [18, 26]. Tumors characterized by basal cytokeratin expression are more probable to have low *BRCA1* expression [25] and harbor *TP53* mutations [14, 31]. Similar with HER2 over-expressing tumors, basal cancers are likely to be of grade 3 tumors [14, 31]. Basal tumors account for 60% to 90% of triple negative cases [27, 38]. These tumors are of particular interest because they follow aggressive clinical course and currently lack any form of standard targeted systemic therapy. Compared with the other subtypes, these tumors are associated with younger patient age, more commonly develop in African-American women and especially among pre-menopausal individuals [39]. Risk factors for this subtype include earlier menarche, high waist-to-hip ratio, and a lack of breast-feeding together with high parity [40]. Unlike the luminal A subtype, where having multiple children and a younger age at the first full-term pregnancy are protective, these factors increase the hazard for basal tumors [41]. These tumors are associated with a lower disease-specific survival and a higher risk of local and regional relapse. Follow-up studies have revealed a time-dependent survival profile for basal breast tumors, with a very poor early outlook diminishing after around 5 years. The metastasis pattern also separates basal tumors from the other breast cancers, with a tendency towards visceral organs (excluding bone) and less likely to involve lymph nodes [41]. The size of basal tumors is, in general, larger than the other subtypes, with a median size of 2 cm in one series [42]. Also, tumors of this class tend to show a rapid growth [41]. Given the triple negative receptor status, basal tumors are not amenable to conventional targeted breast cancer therapies, leaving

chemotherapy the only option in the therapeutic regimen. Two independent studies examining the chemo-resistance of basal cancers have shed light on patients experiencing tumors of this subtype. Their studies converge to the view that these aggressive tumors are sensitive to conventional chemotherapy such as anthracycline and taxane, and their poor prognosis is not driven by the initial chemoresistance but rather to the relatively few treatment options available for triple negative tumors [29]. Besides these conventional therapeutic strategies, many studies have kept suggesting novel targets for basal tumors. It has been suggested that basal tumors may be EGFR-driven [43]. The 'wound response' signature, encompassing genes involved in matrix remodelling and angiogenesis, has been shown to be associated with basal tumors, suggesting other potential avenues of targeting [44].

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Aim of the thesis

Despite the constant progress on the understanding of the molecular bases of cancer, there is still the need to identify novel prognostic and predictive markers aimed at improving diagnosis and, at the same time, designing a more targeted therapeutic approach. For this to be achieved, we have to take into consideration the biological heterogeneity of each tumor that explains different responses to standard therapies harbored by different patients within the same tumor stage and histological type.

Such a combined approach does not apply to improving classification and prognosis of breast cancer only, but it is widely applicable to a better understanding and treatment of all cancers. Nevertheless, it is also crucial to bare in mind that cancer is a genetic disease and molecular analyses aimed at unravelling genetic make-up of tumors are of extreme importance. However, the contribution of the environment in the development of a neoplastic disease should not be ignored, as this would also provide useful insights for cancer prevention. Exposure to harmful substances (i.e. X-rays, UV-light, carcinogens just to list some examples) and to pathogens which might be endemic in some areas could also contribute to triggering genetic alterations whose accumulation may result in malignant transformation, and should not be ignored when trying to understand the etiology of certain tumors.

The aim of this thesis is to provide evidence of how novel genetic markers may contribute to a better classification and diagnosis of cancer, using as examples studies conducted on breast cancer and lymphoma. Also, reactivation of the Epstein-Barr virus due to exposure of unfavorable environmental conditions is provided in another study, to highlight the tremendous importance of the environment in the development of cancer, which is too often dismissed in both the anamnesis of cancer patients and in research studies.

Traditional and new prognosticators in breast cancer: Nottingham index, Mib-1 and estrogen receptor signaling remain the best predictors of relapse and survival in a series of 289 cases

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Abstract

Histopathological and immunohistochemical findings on tissue microarrays, overall survival (OS), disease-free survival (DFS) and incidence of relapses (R) were recorded and statistically analyzed in 289 breast cancers. A higher R and a shorter DFS were significantly related to larger tumors, lymph node invasion, higher tumor grade, absence of estrogen receptors (ER), triple negative tumors and presence of lymphovascular invasion (LVI). Longer OS was observed to be significantly associated with smaller tumor size (T), lymph node negativity, lower tumor grade, absence of LVI, lower Mib-1 expression and with the presence of ER. At multivariate analysis, only T for DFS and lymph node status and triple negativity either for DFS or OS had independent prognostic value. In the 194 lymph node-negative women DFS and OS were inversely related to tumor grade, absence of ER, Mib-1 expression in more than 15% of neoplastic cells and, only for DFs, presence of LVI. In the 95 lymph node-positive the number of involved nodes was the most discriminating parameter either for DFS or OS; T, HER-2 status and presence of LVI were significantly related to DFS. ER negativity was related to higher grade, progesterone receptors (PR) negativity, HER-2 negativity, hence to triple negativity, to basal-like type, Mib-1expression over 15% of neoplastic cells. HER-2 positivity was related to higher grade, ER positivity and PR positivity. Basal-like type was not an independent prognosticator, while triple negative type has a significant relation to shorter OS. The Nottingham prognostic index accurately identifies prognostic groupings

and Mib-1 expression and ER signaling are the key biological predictors even in single cases.

Introduction

Breast cancers are heterogeneous tumors, the vast majority of which originate from the termino-ductular-lobular unit (TDLU) and not from the ductal tree.[1] Nevertheless, they are mainly subdivided into ductal and lobular tumors. Tumors with apparently homogeneous morphology have different genetic profiles. The best characterized of these have been called luminal A, luminal B, HER-2 and basal-like, differing with regard to gene expression, clinical features, response to treatment and prognosis.[2] According to a new cell biology concept, based on gene expression, stem cells (so-called committed progenitor cells) in the human breast can proliferate and give rise to end luminal and myoepithelial lineages.[2] Glandular end cells and cancers originating from them express cytokeratin 8/18, ER, genes associated with ER and PR activation, BCL-2 and GATA 3. These markers are more intensely expressed in some cases, the so-called luminal A, than in other cases, the so-called luminal B variant,[3,4] the latter being more proliferating. The basal compartment and its malignant counterparts, the so-called basal-like cancers, are cells and cancer subtypes mimicking basal epithelial progenitor cells and myoepithelial cells. The majority of them do not express ER, PR and related genes while they strongly express basal cytokeratins,[5,6] proliferation related genes and *TP53* mutations, and sometimes *BRCA1* mutations.[3,7,8] As expected of progenies coming from progenitor cells, there are intermediate cancer cases (CK5+, CK8/18+, ER+ and/or Her2+) towards the glandular end cells.[6] According to various authors,[6,9-11] the co-expression of simple (7, 8, 18 and 19) and stratified (4, 5, 14 and 17) cytokeratins in the same tumors is frequent (from 27–62%), particularly in high-grade tumors. This is not surprising since the basal cytokeratins are

also expressed in luminal cells in the TDLU.[11] There is much agreement on the fact that the basal-like subtype carries a poor prognosis[5,12] and an increased propensity for visceral metastases to the brain and lung[13] although not for locoregional relapse.[14] Her-2+ tumors, independently of being basal-like or not, also have a poor prognosis,[15] as do triple negative cancers (ER-, PR-, Her-2-), which again can be basal-like or not.[16] The main focus of the present study is to help to morphophenotypically characterize the subtypes of breast cancers exhibiting a worse behaviour in a series of 289 patients. There are at least two reasons to do this: first, to find elements to predict the response to currently available treatments, in particular cytotoxic chemotherapy and to try to focus on tumor targets for more appropriate therapy; second, to identify patients at low risk of relapse and poor survival when lymph node-negative, so to have more elements to decide whether chemotherapy can be withheld.[17] In short, the aim is to assist in identifying the correct therapeutic approach in single cases, since the combination of tumor size, lymph node status and tumor grade, i.e., the Nottingham index (NPI) identifies patients with good prognosis, moderate prognosis or poor prognosis.[18]

Table 1. Tumor parameters

Histological tumor type	Number of cases (%)
Ductal NOS	215 (74.4)
Classic lobular	22 (7.6)
Mucinous	18 (6.2)
Apocrine	10 (3.5)
Other	24 (8.3)
Tumor size (cm)	
T1a	14 (4.8)
T1b	47 (16.3)
T1c	137 (47.4)
T2	84 (29.1)
T4b	7 (2.4)
Nodal status	
N0	194 (67.1)
N1a	52 (18)
N2a	28 (9.7)
N3a	15 (5.2)
Grade	
1	49 (17)
2	160 (55.3)
3	80 (27.7)
Lymphovascular invasion	
Absent	198 (68.5)
Present	72 (24.9)
Missing	19 (6.6)

Results

Forty-two out of a total of 289 women with breast cancer died of the disease within 10 y from the diagnosis; the actuarial global OS was 98.5% at 5 y and 84.8% at 10 y. During the study period we recorded 64 relapses of disease, with a median time to progression of 53 mo (range 8–110); the actuarial DFS for the whole population was 87.3% at 5 y and 77.1% at 10 y. A higher R and a shorter actuarial DFS were significantly related to larger tumors ($p < 0.001$), lymph node invasion.($p < 0.001$), higher tumor grade ($p < 0.05$), absence of ER ($p < 0.05$), triple negative type (<0.05) and presence of LVI ($p < 0.001$). The presence of Mib-1 expression in more than 15% of neoplastic cells resulted in a

borderline significance for either higher incidence of R or reduced DFS. Longer OS was observed to be significantly associated with smaller T, absence of lymph node invasion, lower G, absence of LVI, Mib-1 expression in less than 15% of neoplastic cells as well as with the presence of ER. The OS was shorter in triple negative cancers ($p < 0.05$) (Tables 5 and 6). No significant correlation was found between DFS, OS and R and PR, Her-2, EGFR, E-cadherin, VEGF, p53, vimentin, BRCA1 and basal-like histotype (Table 5). At multivariate analysis, only T for DFS and lymph node status and triple negativity either for DFS or OS had independent prognostic value. In triple negative cases, ER negativity was the most discriminating value for worse prognosis, followed by PR negativity and Her-2 negativity. In the 194 women with non-invaded lymph nodes, 18 of whom died of the disease, DFS and OS were inversely related to tumor G, absence of ER, values of Mib-1 expression in more than 15% of neoplastic cells and, only for DFS, presence of LVI. T, PR and Her-2 status, triple negativity, basal-like histotype, as well as all the other markers considered were not discriminating parameters (Table 7). In the 95 patients with lymph node invasion, the number of involved nodes was the most discriminating parameter either for DFS or OS; T, Her-2 status and presence of LVI were significantly related to DFS but not to OS. Triple negativity was very close ($p = 0.06$) but did not reach the level of significance in the correlation to shorter DFS, probably due to a delayed rapid fall of the slope starting from 60 mo of follow-up; however, it had a significant relation to shorter OS ($p < 0.05$). In this group of patients, basal-like tumors had a better prognosis than luminal B cancers ($p < 0.05$). ER negativity (40 cases) was directly related to higher G ($p < 0.001$), PR negativity ($p < 0.001$), Her-2 negativity ($p < 0.005$), hence to triple negativity ($p < 0.001$), to basal-like histotype ($p < 0.001$), Mib-1 expression over 15% ($p < 0.001$), EGFR negativity ($p < 0.001$), p53 positivity ($p < 0.05$) and vimentin positivity ($p < 0.05$). It was not significantly related to T, lymph node status, LVI, E-cadherin, VEGF and BRCA1. Her-2 positivity (73 cases) was directly related to higher G ($p < 0.001$), ER

positivity ($p < 0.005$) and PR positivity ($p < 0.005$); it tended to be associated with larger tumors although without reaching the significance level ($p = 0.06$). Her-2+ and Her-2- cases were allocated in the same percentage in the basal-like group of cancers36 but, compared with the luminal A and B groups, Her-2+ tumors were significantly more numerous in the basal-like group ($p < 0.005$). G3 cancers ($p < 0.05$), EGFR+ tumors ($p < 0.001$) and triple negative ($p < 0.001$) cancers were more numerous in the basal-like group, while ER+ tumors were less numerous ($p < 0.001$) than in the luminal A and B groups. Her-2- cancers had a significantly higher Mib-1 expression ($p < 0.01$), were more frequently EGFR- ($p < 0.005$) and p53+ ($p < 0.01$), and had a higher G ($p < 0.001$).

Table 2. Biological characteristics of the study population (289 patients)

		Number (%)
Estrogen receptors	Absent	40 (13.8)
	Present	249 (86.2)
Progesteron receptors	Absent	61 (21.1)
	Present	228 (78.9)
Her-2	Negative	216 (74.7)
	Positive	73 (25.3)
Triple negative	No	268 (92.7)
	Yes	21 (7.3)
Immunophenotype	Basal-like	36 (12.4)
	Luminal A	222 (76.8)
	Luminal B	31 (10.8)
Mib-1	≤15%	196 (67.8)
	>15%	74 (25.6)
	Missing	19 (6.6)
CD44	Absent	167 (57.8)
	Present	86 (29.8)
	Missing	36 (12.5)
E-Cadherin	Absent	20 (6.9)
	Present	186 (64.4)
	Missing	83 (28.7)
EGFR	Absent	21 (7.3)
	Present	241 (83.4)
	Missing	27 (9.3)
VGFR	Absent	201 (69.6)
	Present	58 (20.1)
	Missing	30 (10.4)
p53	Absent	203 (70.2)
	Present	23 (8)
	Missing	63 (21.8)
BRCA-1	Absent	205 (70.9)
	Present	8 (2.8)
	Missing	76 (26.3)
Vimentine	Absent	213 (73.7)
	Present	7 (2.4)
	Missing	69 (23.9)

Discussion

In our series of cases, DFS and R were significantly correlated to T, lymph node status, G, LVI as morphological factors and to ER signaling. Longer OS was significantly associated with smaller T, lymph node negativity, lower G, no LVI, and ER positivity and Mib-1 expression in less than 15% of neoplastic cells. In the patients without lymph node invasion, T lost its influence on survival, while lower G, ER positivity and Mib-1

expression in less than 15% of neoplastic cells were associated with longer OS and DFS and with lower R. In patients with lymph node invasion, the most discriminating parameters were the number of involved lymph nodes and the tumor size. In this group of cases, luminal B cancers had a worse prognosis than luminal A and basal-like tumors, while triple negativity had a significant relation to shorter OS. Therefore, we can say that, in agreement with Wirapati et al.19 NPI20 accurately identifies prognostic groupings. Proliferation, as tested by Mib-1 expression, and ER signaling were the key biological predictive parameters in our cases. In contrast with Wirapati et al.,19 these parameters did not include Her2. In our cases, Her-2 was not discriminating by itself; however, Her-2 negativity added to the discriminating negative power of ER negativity and PR negativity. In our series, as in others',[14,21,22] triple negative cases as a group had a worse prognosis; however, they were a heterogeneous category including cancers with excellent prognosis (such as the medullary histotype) and cancers with poor prognosis.[23,24] Belonging to this group does not identify the prognosis of single cases.[25] We also analyzed the so-called basal-like cancer "problem." Basal-like cancers originate from the TDLU and not from myoepithelium since cytokeratins 5, 14 and 17, which decorate basal cells in stratified epithelia, are also expressed by luminal cells.

Luminal cells express cytokeratins 8, 18 and 19, which are also expressed by basal-like cancers, even in our cases, but not by myoepithelial cells.11 Nielsen et al.12 identify basal-like tumors by gene-expression profiling with four markers (ER negativity, Her-2 negativity, EGFR positivity, CK 5/6 positivity) obtaining a specificity of 100%. Cheang claims[26] that five markers (ER, PR, Her-2, EGFR and CK 5/6) are needed, while Moinfar points[27] out that CK 5 and CK 17 are discriminating. Rakha and Ellis affirm[16] that in their experience an acceptable degree of specificity and sensitivity is reached using at least two basal markers among CK 5/6, CK 14, CK 17 and EGFR. In the present study we followed Rakha and Ellis's indication, independently of the positivity or negativity of any other

parameter. There is also a tendency to define basal-like cancers as those tumors that are ER-, PR- and Her-2 -, i.e., the so-called triple negative cancers.[28] However, 14–45% of basal-like cancers express at least one of these markers.[12,14,16,21,29-34] Therefore, it is clear that basal-like cancers are still to be unequivocally defined at the immunohistochemical level, and that expression of basal cytokeratins is not the unique requirement. From 3–20% of cancers are basal-like.[6,17,35,36] In our series, 36 out of 289 tumors (12.4%) were basal-like. The large majority of them were G3, with a prevalently solid architecture, a dense population of cells, a well-defined, pushing border of invasion and the absence of association with vascular invasion or lymph node involvement (as stressed by Fulford et al.[37]). They had sometimes a high mitotic and apoptotic rate, geographic necrosis, sometimes spindle or squamous metaplastic changes, glomeruloid microvascular proliferation and stromal lymphocytic response (as in the cases of Fulford et al.[37], Langerod et al.[38] and Diallo-Danebrock et al.[39]). Our ER- and triple negative cancers were histologically similar to basal-like cancers. Her-2+ cancers had more frequently infiltrative borders. This finding is also very much in agreement with other authors' observations (reviewed in ref. 16). We did not find any correlation of basal-like cancers with OS, DFS and R; however, in the group of cases with invasion of lymph nodes, basal-like tumors tended to be more represented than luminal cancers although the difference was not significant. This is in disagreement with the results of other authors;[13] possible reasons for this disagreement could be as follows: low number of basal-like cancers in our series; use of different antibodies and staining techniques; lack of reliable quality control when we dealt with negativity instead of positivity; lack of consensus regarding the definition of ER and Her-2 positivity;[40] not enough representative tissue arrays given the heterogeneity of breast cancers; difficulty in establishing a threshold of positivity for ER, PR and Her-2; the fact that basal-like tumors often express CK 8 and 18 as luminal tumors do.[16,18] In lymph node-negative cases (67%), T lost its discriminating power and only G, ER- and

Mib-1 expression in more than 15% of neoplastic cells were significantly related to shorter OS. This reinforces the concept that proliferation and ER signaling are the best biological prognosticators, particularly in lymph nodenegative cases.[19,41,42] Triple negative cases of the present series had a poor prognosis as a group and the negative discriminating value of ER negativity and PR negativity was reinforced by Her-2 negativity, probably because the benefit of targeted therapy was lacking in Her-2 negative cases.[16] Her-2 positivity was not associated with shorter OS and DFS probably because the negative effect of its amplification is balanced by the use of targeted therapy. In conclusion, our results are in agreement with the conclusion of Gusterson[18] that NPI is the most reliable method of predicting survival of operable breast cancers and that the genes related to proliferation[41] and to ER signalling[42] are the best biological prognosticators even in single cases. In lymph node-negative cases, the most predictive parameters indicating a worse prognosis and therefore a correct therapeutic approach are ER negativity and Mib-1 expression in more than 15% of neoplastic cells. Among the subtypes, only the triple negative type is an independent indicator of a worse prognosis, because ER negativity is associated with Her-2 negativity. Hence, as Gusterson affirms,[18] it is premature to conclude (reviewed in ref. 43) that histological subtypes, as identified by gene expression, are the best prognosticators.

Table 3. Morphological features of invasive basal-like, triple negative, Her2$^+$ and ER$^-$ cancers

Morphological features	Basal-like (n = 36)	Triple negative (n = 21)	Her2$^+$ (n = 73)	ER$^-$ (n = 40)
Geographic necrosis	26/36 (72%)	13/21 (62%)	6/73 (8%)	30/40 (75%)
Pushing border	23/36 (64%)	14/21 (67%)	4/73 (5%)	23/40 (57%)
Infiltrative border	13/36 (36%)	7/21 (33%)	69/73 (95%)	17/40 (43%)
Lymphoid stroma	19/36 (53%)	10/21 (48%)	8/73 (11%)	20/40 (50%)
Apocrine features	0/23 (0%)	0/21 (0%)	1/73 (1%)	2/40 (5%)
Metaplastic features	3/36 (8%)	1/21 (5%)	2/73 (3%)	1/40 (2%)
Squamoid features	5/36 (14%)	5/21 (24%)	0/73 (0%)	2/40 (5%)
Central fibrosis	6/36 (17%)	2/21 (10%)	10/73 (14%)	4/40 (10%)

Table 4. Primary antibodies

Antibody	Source	Clone	Dilution
CK 5/6	Dako	D5/16B4	1:70
CK 14	Bio-Optica	LL002	1:50
CK 8	DBA-Italia	C-51	
CK 18	Menarini	DC-10	1:500
ER	Bio-Optica	SP1	1:100
PR	Bio-Optica	SP2	1:50
Her2	Dako	Policlonal	1:600
Mib1	Bio-Optica	SP6	1:200
E-cadherin	Dako	NCH-38	1:50
EGFR	Zymed	31 G7	1:50
VEGF	Dako	VG1	1:50
p53	Bio-Optica	pAb 240	1:200
BRCA1	Hystoline	GLK-2	1:50
Vimentine	Dako	V9	1:300

Materials and Methods

Patients. Patients submitted to surgery for invasive breast cancer at the Department of Human Pathology and Oncology of the University of Siena, between January 1993 and December 1998 were included in the present observational study. We excluded patients with distant metastases at the time of diagnosis, patients who received a neoadjuvant chemotherapy and patients who did not follow a standardized program of clinical and instrumental follow-up at our outpatient clinic, as well as patients who had no sufficient paraffin-embedded tissue available for tissue microarrays (TMA). The study population included 289 female patients with a mean age of 61 ± 12 y (range 32–82); 218 (75.4%) women were postmenopausal. Surgical treatment consisted of modified radical mastectomy in 144 cases and partial mastectomy in 145 cases; all patients received a level II axillary dissection, with a mean number of 16 + 5 lymph nodes removed (range 8–37). Postoperative radiation therapy to the residual breast was administered in all cases of

partial mastectomy. Adjuvant postoperative therapy was administered to 215 patients, with 102 women receiving chemotherapy and 143 receiving hormone therapy, 113 as an exclusive treatment and 30 following a primary adjuvant chemotherapy. Patients were evaluated every 6 mo for the first five y, and then annually; mean follow-up was 113 mo (range 18–120). The data on patients OS, DFS and R were recorded. Histopathology. Histopathological findings were reviewed. For each case, histopathology defined the cancer histotype, pathological stage assessed according to the criteria established by the International Union Against Cancer, grade according to the modified ScarffBloom-Richardson criteria, presence of peritumoral LVI (Table 1). Tumors were also evaluated for biological characteristics (Table 2) and the presence of geographic necrosis, border appearence, lymphocytic stromal response, apocrine features, metaplastic features, basaloid cell change and large central acellular zone (Table 3). The evaluation was limited to the invasive portion of the tumor. Immunohistochemical analysis on TMA. For each case, a representative area of the tumor was selected based on HE-stained sections from paraffin blocks. A hollow needle was used to remove 1 mm tissue cores (needles with varying diameters of 0.6 up to 2 mm were available) from these areas. An HE-stained slide arranged on the donor block surface was used for orientation. These tissue cores were then transferred to a recipient paraffin wax block into a ready-made hole, guided by a defined x-y position in a precisely spaced array pattern. 4 mmicron-thick sections were cut from this block using a microtome, mounted on a microscope slide and stained by immunohistochemistry. The characteristics of the antibodies used are listed in Table 4. Following deparaffinization in xylene, slides were rehydrated through a graded series of alcohol and placed in running water. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase and methanol. Samples were steamed for antigen retrieval with 10 mM citrate buffer (pH 6.0) for 35 min. Following protein block, slides were incubated with antibody and washed with normal swine serum in Tris buffer saline (TBS). 3,3'-

Diaminobenzidine tetrahydrochloride (DAB) was used for the visualization of the antibody/enzyme complex. Slides were counterstained with Harris's hematoxylin and examined by light microscopy. Assessment of staining was based on a semiquantitative approach and tumor immunoreactivity was scored as follows: 0 = negative, 1 = weak positive, and 2 = moderate/strong positive in combination with the percentage of cells showing positive staining. ER and PR were scored positive if at least 5% of neoplastic cells showed nuclear staining. Her-2 was scored positive if a 2+ or 3+ result was found. Details of Mib-1 expression by means of immunohistochemistry and choice of cut-off levels are reported elsewhere.[44] In brief, patients were stratified according to different percentages of Mib-1 expression staining and cut-off point analysis of DFS and OS was used to select the cut-off value for Mib-1 expression positivity (data not shown). The best cut-off point for semiquantitative Mib-1 expression that maximized the separation of the survival curves was 15% of neoplastic cells, which was therefore selected as the cut-off value. For evaluation of immunohistochemical staining, positive controls and normal breast tissues were used. For Her-2, p53 and EGFR a known positive external control was utilized. Different cores of the same tumor were scored individually, then the mean of the readings was calculated, once the uninformative cores were eliminated. Stainings were scored as positive or negative independently of their intensity. The observers

(Megha T and Malagnino V) scored each staining pattern without previous knowledge of the outcomes on two separate occasions and a good intra- and interobserver correlation between the results was found. Statistical analysis. Correlation between clinicopathological variables was investigated by means of univariate analysis. The chi-square test was used to assess the statistical significance of the association between categorical variables. DFS and overall survival OS were calculated from the date of surgery; follow-up was closed at 10 y. We evaluated the prognostic significance of the different variables with respect to DFS and OS by means of the log-rank test and

compared such prognostic significance to that of the other clinical and pathological factors considered by means of Cox regression analysis. The Statistical Package for the Social Sciences software (version 11.0) (SPSSTM, Chicago, IL USA) was used.

Table 5. Correlations between different parameters and incidence of relapses

		Relapse n (%)	p
pT	T1a	1/13 (7.1)	
	T1b	8/47 (17)	
	T1c	23/137 (16.8)	
	T2	26/54 (31)	
	T4b	6/7 (85.7)	**<0.001**
pN	N0	28/194 (14.4)	
	N1a	13/52 (25)	
	N2a	12/28 (49.2)	
	N3a	11/15 (73.3)	**<0.001**
Tumor grade	1	8/49 (16.3)	
	2	31/160 (19.4)	
	3	25/80 (31.3)	**<0.05**
ER	Negative	14/40 (35)	
	Positive	50/249 (20.1)	**<0.05**
PgR	Negative	14/61 (23)	
	Positive	50/222 (22.5)	0.944
Her2	Negative	52/216 (24.1)	
	Positive	12/73 (16.4)	0.174
Triple negative	Yes	9/21 (42.9)	
	No	55/268 (20.5)	**<0.05**
Immunophenotype	Basal	7/36 (19.4)	
	Luminal A	52/222 (23.3)	
	Luminal B	5/31 (16.1)	0.695
LVI	Absent	32/198 (16.2)	
	Present	29/72 (40.3)	**<0.001**
Mib-1	≤15%	36/196 (18.4)	
	>15%	21/74 (28.4)	0.07
CD44	Absent	38/167 (22.8)	
	Present	19/86 (22.1)	0.905
Caderina E	Absent	5/20 (25)	
	Present	41/186 (22)	0.763
Egfr	Absent	55/241 (22.8)	
	Present	6/21 (28.6)	0.550
Vegfr	Absent	15/58 (25.9)	
	Present	45/201 (22.4)	
p53	Absent	48/203 (23.6)	
	Present	3/23 (13)	0.249
BRCA1	Absent	49/205 (23.9)	
	Present	1/8 (12.5)	0.455
Vimentina	Absent	51/213 (23.9)	
	Present	0/7	0.140

Table 6. Prognostic parameters for DFS and OS at univariate analysis on the whole population (289 patients)

		5 y DFS %	10 y DFS %	p	5 y OS %	10 y OS %	p
pT	T1a	100	92.9		100	92.9	
	T1b	91.2	83.2		97.8	93.3	
	T1c	88.8	82.8		98.5	86.4	
	T2	82.9	67.7		98.7	77.3	
	T4b	57.1	19	**<0.001**	83.3	62.5	**<0.05**
pN	N0	91.6	85.2		99.5	90.4	
	N1a	89.8	72.7		97.9	85.1	
	N2a	71	56.1		92.6	61.7	
	N3a	53.3	26.7	**<0.001**	93.3	53.3	**<0.001**
Grade	1	93.8	83.3		97.9	91.5	
	2	87.3	80.1		99.4	87	
	3	83.3	67.1	**<0.05**	97.4	75.4	**<0.05**
ER	Absent	82.5	65		95	72.5	
	Present	88	79.2	**<0.05**	99.2	86.8	**<0.05**
Triple negative	Yes	80.9	57.1		95.2	66.7	
	No	87.8	78.8	**<0.05**	100	86.3	**<0.05**
LVI	Absent	91.3	83.3		99.5	88.4	
	Present	75.6	57.8	**<0.001**	97	71.7	**<0.01**
Mib-1	≤15%	90.2	81.1		98.9	89.3	
	>15%	84.8	70.7	0.06	97.2	77.4	**<0.05**

Table 7. Prognostic parameters for DFS and OS at univariate analysis in lymph node negative patients (194 patients)

		5 y DFS (%)	10 y DFS (%)	p	5 y OS (%)	10 y OS (%)	p
Tumor grade	1	97.4	94.7		100	100	
	2	93.4	88.6		99	93.3	
	3	82.9	69.5	**<0.005**	97.8	75.6	**<0.0005**
ER	Absent	87.5	70.8		95.8	79.2	
	Present	92.2	87.3	**<0.05**	99.4	92	**<0.05**
LVI	Absent	92.7	87.2		99.3	99.1	
	Present	84.6	73	**<0.05**	96.15	80.7	0.11 n.s.
Mib-1	≤15%	96.3	90.2		99.2	95.4	
	>15%	83.6	76.5	**<0.05**	97.6	80.9	**<0.005**

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EBV reactivation and chromosomal polysomies: *Euphorbia tirucalli* as a possible co-factor in endemic Burkitt lymphoma

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ABSTRACT

Burkitt lymphoma is endemic in the Equatorial Belt of Africa and accounts for up to 70% of childrens' cancer. The molecular hallmark of this tumor is a deregulated, activated, *MYC* gene mostly due to a chromosomal translocation, though alternative pathogenetic mechanisms leading to *MYC* activation have also been described. Especially in its endemic clinical variant, Burkitt lymphoma is associated with the oncogenic Epstein-Barr virus (EBV), and holoendemic malaria acts as an amplifier. The combination of malaria and early infection with the Epstein-Barr virus is thought to play an important role in inducing this tumor in the lymphoma belt of Africa. Environmental factors may also cooperate in Burkitt lymphomagenesis in the endemic regions, such as plants used as traditional herbal remedies.

Euphorbia tirucalli, a plant known to possess EBV-activating substances, has a similar geographical distribution to endemic Burkitt's Lymphoma and is used as a hedge, herbal remedy and toy in the Lymphoma Belt. A single report of more than twenty years ago describes the ability of *E. tirucalli* extracts to induce chromosomal abnormalities when added to EBV-infected cord blood B-lymphocytes. Since then, no further study has been performed to clarify the relationship between *E. tirucalli* and the induction/incidence of BL in the endemic areas, neither information about the molecular mechanisms has been provided.

Therefore, in this study we aimed at determining if exposure to *Euphorbia tirucalli* could contribute to lymphomagenesis, and at which extent. Lymphoblastoid and cord blood-derived cell lines were treated with plant extracts, and the expression of EBV-coded proteins was checked, to assess EBV reactivation. The occurrence of chromosomal translocations was then investigated by FISH.

Cells treated with *E. tirucalli* showed a modulation of EBV latency genes, as increasing doses of *E. tirucalli* determined the expression of Zebra and of the EBV early antigen (EA). In addition, a marked over-expression of LMP1 was detected together with up-regulation of EBNA1 and EBNA2.

A 5-day treatment induced polysomies for chromosome 8, and though no chromosomal translocations for *MYC* were detected, a consequent up-regulation of c-MYC expression was observed. In addition, treatment with plant extracts determined BCL-2 over-expression.

Our preliminary results suggest that *E. tirucalli* is able to reactivate EBV and determine chromosomal alterations, which leads to c-MYC altered expression. The existence of genomic alterations might determine the accumulation of further genetic alteration, which could eventually lead to a transformed phenotype.

Introduction

Burkitt's Lymphoma (BL), a high-grade Non-Hodgkin's lymphoma, is endemic in the Lymphoma Belt of Africa, which lies between 10° N and 10°S of the Equator (1, 2). Within these geographical boundaries BL accounts for up to 70% of childrens' cancer with rates up to 10 cases of Burkitt's Lymphoma per 100,000 children under the age of 14 years (3). Burkitt's Lymphoma characteristically has a translocation involving a deregulated, activated, *MYC* gene on chromosome 8 and immunoglobulin genes on chromosome 14, or, more rarely, chromosomes 2 or 22 (1), though alternative pathogenetic mechanisms leading to *MYC* activation have also been described (4,5). Burkitt's Lymphoma is associated with the oncogenic Epstein-Barr virus (6), in particular 98% of Burkitt's Lymphoma cases in the Lymphoma belt show positivity to EBV (7). EBV is recognised as a Class 1 human carcinogen and is thought to play a pivotal role in lymphomagenesis in endemic Burkitt's Lymphoma (8). Holoendemic malaria acts as an amplifier and has been shown to be able to activate the latent EBV in B-lymphocytes in children in the Equatorial Belt (9, 10). The combination of malaria and early infection with the Epstein-Barr virus is thought to be responsible for boosting the incidence of Burkitt's lymphoma a hundred-fold in Africa, compared with rates in the United Kingdom, France and the USA (11,12). Children who subsequently develop the endemic Burkitt's Lymphoma have raised antibody levels to the EBV Viral Capsid Antigen (VCA) of EBV several years before they actually develop the tumour (13). Raised levels of this antibody are also found in the relatives of children with Burkitt's Lymphoma (14) and in those who have used traditional herbal remedies (15), which have been shown to be capable of activating the EBV (16).

Euphorbia tirucalli, a plant known to possess EBV-activating substances has a similar geographical distribution to endemic Burkitt's Lymphoma (17) and is used as a hedge,

herbal remedy and toy in the Lymphoma Belt. This plant is found significantly more often at the homes of Burkitt's Lymphoma patients (17, 18) and the incidence of Burkitt's Lymphoma has fallen in Northern Zambia following the eradication of thickets of *E. tirucalli* (19).

Euphorbia tirucalli possesses a milky, rubbery sap which contains a 4-deoxyphorbol ester (20) closely-related to the tumour-promoter substance TPA (12-O-tetra-decanoy-phorbol-13-acetate), which is derived from another Euphorbia, *Croton tiglium*. These TPA-related extracts of *Euphorbia tirucalli*, present in the plant are secreted into the soil around the plant in active form (21) and can activate the latent EBV within the cell (16, 22), enhance EBV-mediated cell transformation (21), and modulate EBV-specific T-cell activity (21), myelopoiesis and cellular immunity (23). EBV and TPA can work synergistically in nude mice to produce both T- and B-cell lymphomas (24). TPA's activity is also potentiated by the association with nucleic acids. Other plants commonly found in the Lymphoma belt and elsewhere in the tropics, typically those belonging to the Euphorbiaceae, and Thymelaeaceae families are also known to induce the EBV lytic phase (16). Some, but not all of these Euphorbiae, contain identical or similar phorbol esters to those found in *E. tirucalli* (25). EBV-activation has been shown to be dependent on cellular protein kinase C (PKC), irrespective of the extracts' tumour-promotion abilities (26). The TPA-related substances present in *E. tirucalli* could be expected to exhibit similar properties to those of TPA.

A research paper of the early nineties reported that *E. tirucalli* extracts can induce chromosomal abnormalities when added to EBV-infected cord blood B-lymphocytes (27). Aya et al. showed that the cells multiplied rapidly following exposure to the extracts and, after one year of culture, ten per cent of the chromosomal abnormalities induced by these plant extracts affected chromosome 8 with activation of the oncogene *MYC*, thus

reproducing the crucial translocation characterizing Burkitt's Lymphoma (27), and that cells treated with the Euphorbia extracts produced lymphomas when injected into nude mice (27).

These observations suggest that *Euphorbia tirucalli* extracts and, possibly, extracts of other plants, which are known to have similar EBV-activating properties, in conjunction with other environmental factors, could play an important role in lymphomagenesis in endemic African Burkitt's Lymphoma. Despite the interesting observation reported by Aya in the early nineties, no further study since then has elucidated the molecular mechanisms by which some plant extracts may act as a cofactor in lymphomagenesis. Therefore, it is also interesting trying to explain the close link between the geographic distribution of *E. tirucalli* and the incidence of BL in the endemic areas. The aim of our study was to determine if exposure to *E. tirucalli* extracts could result in EBV reactivation and induction of genomic alteration, which might contribute to transformation. Therefore, we treated both a lymphoblastoid cell line (LCL) and a cord-blood (CB) derived cell line newly infected with EBV with this plant extracts. It was considered that the LCLs are likely to have accumulated pre-existing genetic abnormalities (28) having been cultured for a number of years, whereas the cord-blood cell line should be a better *in vitro* model to mimic the effects of the plant extracts *in vivo* on EBV-infected cells. Cells were treated with different concentrations of *E. tirucalli*, and we monitored its effect on cell proliferation, EBV antigen expression and induction of genomic alterations, such as chromosomal translocations.

Our results indicate that exposure to the plant extracts is able to reactivate EBV from its latent phase, as indicated by the expression of the EBV Zebra antigen following the treatment. In addition, the expression of EBV-early antigens was also observed, along with a marked up-regulation of LMP1, EBNA1 and EBNA2. In addition, though the

specific chromosomal translocations present in Burkitt's lymphoma were not detected after *E. tirucalli* exposure, we observed the occurrence of polysomies involving chromosome 8, as demonstrated by the existence of multiple signals for *MYC* by FISH, which may result in over-expression of c-MYC, both at the mRNA and the protein level. In addition, increased expression for BCL2 was also observed, even in the absence of any genetic translocations involving this gene.

All together, our results suggest that *E. tirucalli*, through EBV reactivation and induction of genetic alterations leading to *MYC* over-expression, could contribute to the malignant transformation process.

MATERIALS AND METHODS

Cell lines and cell culture

The human lymphoblastoid cell line (LCL) was a kind gift of Prof. A. Lanzavecchia (IRB, Bellinzona, Switzerland). The human cord-blood (CB) derived cell line was obtained from cells newly infected and immortalized by EBV, following the protocol described by Pelloquin et al (29). Mononucleated cells were isolated from cord-blood by Ficoll fractionation. After isolation, purified EBV obtained from B95.8 cell line (kindly provided by dr. M. Kleines, Austria) was added to the cell culture, in a 1:1 ratio. Cells were cultured in the presence of Cyclosporin A at a final concentration 2μg/ml. The efficiency of infection was demonstrated by cluster formation in the cell culture after an overnight incubation with the virus. The establishment of the cell line was achieved after one month. Immunophenotype of the established CB cell line ($CD79^+$, $CD34^-$, $CD138^-$, $IRF4^-$, $BCL2^+$ and $BCL6^-$) was assessed as described below. Before treatment, cell caryotype was also assessed to confirm the absence of chromosomal translocations and aneuploidies. An EBV-negative Burkitt lymphoma-derived cell line (Ramos) was also used, and treated as follows. For daily experiments and treatment with *E. tirucalli* cells were cultured in RPMI supplemented with 10% FBS, 1% L-glutamine, penicillin/streptomycin, with 5% CO_2, at 37°C.

Immunocytochemistychemistry

Immunocytochemical studies (ICC) were performed on representative cell smears of both treated and untreated cells using microwave pre-treatment or proteolytic digestion of slides for antigen retrieval. A large panel of antibodies (Table 1) recognizing the various EBV antigens was applied, in conjunction with the streptavidine-peroxidase method

(Ultravision Detection System Anti-Polyvalent, HRP by Lab Vision Corporation and Liquid DAB Substrate Chromogen System by DAKO), to visualize antibody binding. Protein expression was then quantified by counting the percentage of positive cells per HPF in 10 randomly chosen HPFs.

E. tirucalli treatment and cell proliferation

E. tirucalli plant extracts were prepared as described by Ito (30). Briefly, *E. tirucalli* extracts were obtained using 200 ml ether under reflux for 72 hours. The ethereal solution was then evaporated down and the resultant oily extracts were then dissolved in methanol, and served as the test substance for EBV antigen activation. Cells were treated with different concentration of *E. tirucalli* (0.1, 0.5, 1 and 10 μg/ml), resolved in methanol. As a normal control, untreated cells were cultured with the same amount of methanol. For the proliferation assay, cells were counted each day for 4 days. Statistical significance was assessed by the analysis of variance (ANOVA) test. For detection of EBV-specific responses, Ramos cells, an EBV-negative Burkitt lymphoma-derived cell line, were used as negative control, and were cultured as previously described.

Cell death analysis

Cell death was evaluated by several approaches. Cell viability was checked by Trypan Blue exclusion test. Cell cycle analysis was performed by flow cytometry on a FACStar (BD Bioscience, CA). Forward Scatter (FSC) and Side Scatter (SSC) signals were recorded in linear mode. Dead cells and debris were gated out using scatter properties of the cells and additionally using propidium iodide (PI) at a concentration of 1 μg/ml. Data was analyzed using CellQuest software (BD Bioscience, CA). Apoptosis was detected by DNA laddering on a 1% agarose gel. Caspase staining for caspase 3 and 8 was detected by immunocytochemistry, as previously described.

Fluorescence in situ hybridization (FISH)

Briefly, *MYC* and *BCL2* rearrangements were sought using the *MYC* FISH DNA Probe–Split Signal using standard procedures (*BCL2*, IgH, IgL, *BCL6*, break-apart probes and *MYC* dual color probe). Briefly, smeared cells were air-dried, immersed in a jar filled with pre-treatment solution, and warmed at 98°C for 10 min by means of a Whirlpool JT 356 microwave. Subsequently, the slides were cooled for 15 min at RT. After two passages of 3 min each in Wash Buffer, excess buffer was tapped off and the slides digested with cold Pepsin for 20 min in a Dako Cytomation Hybridizer (Dako, Denmark). The slides were then washed twice in Wash Buffer for 3 min, dehydrated using increasing graded ethanol series, air-dried, and finally 10 µl of probe mix were applied to each tissue section. The slides, covered with coverslip and sealed with rubber cement, were then incubated in the DakoCytomation Hybridizer (Dako, Denmark) according to the manufacturer's recommendations. The next day, slides were treated with stringency buffer at 65°C for 2 min, then placed twice in Wash Buffer for 3 min, dehydrated using increasing graded ethanol series, air-dried, and counterstained applying 15 µl of Fluorescence Mounting Medium. Hybridization signals were visualized using a Leica microscope equipped with a triple-band filter for detecting green fluorescent protein (GFP)/spectrum green, Texas red/spectrum orange, and DAPI/spectrum blue. Images were captured and archived using Leica FW4000 software. One hundred non-overlapping interphase nuclei were scored for each tumor specimen. In normal nuclei, two yellow fusion signals (2F) are detected, whereas in nuclei with translocations, a yellow (or red-green juxtaposed) signal is obtained from one red and one green segregated signal (1F1R1G). The results were further confirmed by additional FISH analysis using split-signal probes for IgH and IgL loci as well as an LSI *IGH*/*MYC* CEP 8 Tri-color dual-fusion probe (Vysis, Abbott Molecular IL, USA) specific for the detection of the translocation t(8;14). All reagents, instruments and split-signal probes were kindly provided by DakoCytomation (Glostrup, Denmark). To

specifically detect chromosome 8, the centromeric probe Zyto Light SPEC CMYC/CEN8 Dual Color Probe (ZytoVision, Germany) has been used. To establish the *MYC*:chromosome 8 ratio, 100 nuclei were randomly chosen and signals for *MYC* and chromosome 8 were counted.

qRT-PCR

Real-time PCR for *MYC* and *BCL2* was performed using FluoCycle SYBR green (Euroclone, Celbio, Italy) according to the manufacturer's instructions and *HPRT* as an internal control.

Primer sequences for *MYC* amplified a region of 129bp: LEFT: AGCGACTCTGAGGAGGAAC; RIGHT: TGTGAGGAGGTTTGCTGTG. Primer sequences for *BCL2* amplified a region of 258bp: LEFT: 5'-TTGCCACGGTGGTGGAGGA-3'; RIGHT: 5'-ACAGCCAGGAGAAATCAAACAG-3'. Primer sequences for *HPRT* amplified a region of 191bp: LEFT: AGCCAGACTTTGTTGGATTTG; RIGHT: TTTACTGGCGATGTCAATAAG. Differences in gene expression were calculated using the ΔΔCt method (31).

Indirect immunofluorescence

For c-MYC detection, cells were smeared on positively charged slides after *E. tirucalli* treatment (Day 5) and fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature. Permeabilization was achieved by washing cells in PBS, 0.2% Triton X-100 and 1% BSA. Saturation was performed for 1 hour at room temperature in goat serum (Zymed laboratories, Milan, Italy). All of the antibodies were diluted in goat serum. Primary antibody incubation was carried out at room temperature for 1 hour, using anti-c-MYC (9E10 sc-40: Santa Cruz Biotechnology, Santa Cruz, CA) 1:50. Secondary goat anti-mouse antibody, conjugated with Alexafluor568 (Molecular Probes, Invitrogen, Milan, Italy), was diluted 1:100 in goat serum and incubated at room temperature for 45

minutes. The slides were examined on an Axiovert 200 microscope (Carl Zeiss, Germany) and processed with Zeiss software (Carl Zeiss, Germany). Nuclei were counterstained by DAPI.

RESULTS

E. tirucalli treatment affects cell proliferation

We treated LCLs and the cord blood-derived cell line with increasing concentrations of *E. tirucalli*, as reported in Methods, using methanol-treated cells as a control. The effects of the treatment on cell proliferation and cell death were monitored. A dose-dependent reduction in cell proliferation was observed after *E. tirucalli* treatment (FIGURE 1a), accompanied by high rates of cell deaths (FIGURE 1b).

Cell death seemed to be due to necrosis rather than to apoptosis, as no DNA laddering nor caspase activation was observed following *E. tirucalli* treatment (FIGURE 1c-d). To assess whether cell death was due to the toxicity effects of the plant extracts or to reactivation of EBV, we treated an EBV-negative cell line using the same experimental conditions, and we observed a similar reduction in cell proliferation accompanied by an increase in cell death suggesting that cell death was due to plant toxicity. (FIGURE 2)

E. tirucalli modulates the expression of EBV-antigens

There are three different latency programs of EBV, characterized by the differential expression of its coded genes (32). In additions, the expression of some EBV-genes, such as Zebra, indicates the shift from the latent to the lytic phase of the virus (33). Reactivation of EBV as a consequence of TPA-analogous treatment had been reported by literature (24). We therefore treated cells with different concentrations of *E. tirucalli* and monitored the expression of EBV-coded genes by ICC. After *E. tirucalli* treatment, we observed the expression of Zebra, which was not expressed by untreated cells. In addition, enhanced expression of the EBV early antigen (EA), LMP1 and EBNA2 expression was also observed, whereas no significant variation of the EBV early antigen gp350 was detected following *E. tirucalli* treatment. Higher concentrations of plant extracts (10

μg/ml) resulted in a more marked EBV protein expression. Comparable results were obtained in LCL and cord-blood cell lines. Table 2 summarizes immunocytochemical results obtained in treated vs. untreated cells. FIGURE 3 shows ICC results.

E. tirucalli induces chromosome 8 polysomy

E. tirucalli has been shown to induce genetic alterations, particularly those involving the oncogene *MYC* (27). To detect the onset of specific chromosomal translocations after *E. tirucalli* treatment, we performed FISH analysis to identify the most frequently described chromosomal translocations occurring in aggressive B-cell lymphomas, using probes designed to detect *MYC*, *BCL-2, BCL6* and their respective Ig partners. Using this approach, no balanced translocations involving *BCL2, BCL6,* IgH and IgL were detected.

Multiple signals for *MYC* were detected in about 17% of cells, ranging between 3 and more copies, in contrast to the normal 2 copies, though no specific chromosomal translocation could be identified. To assess whether these signals were dependent on gene amplification or to a chromosome 8 polysomy, we used a centromeric probe to detect both *MYC* and centromere signals. Our results indicated a chromosome 8 polysomy, as more than 2 signals for the centromeres were detected, together with multiple signals for *MYC*. Table 3 summarizes FISH results. FIGURE 4 summarizes FISH results for *BCL2* and *MYC*.

c-MYC expression is up-regulated following _E. tirucalli_ treatment

The presence of multiple copies of *MYC* is compatible with an over-expression of the c-MYC protein, as occurs in Burkitt lymphoma. Therefore we checked the expression level of c-MYC by qRT-PRC and immunofluorescence. As a consequence of chromosome 8 polysomy, up-regulation of c-MYC was observed, as expected, at both levels (FIGURE 6 a-b).

BCL2 is overexpressed following *E. tirucalli* treatment

BCL2 deregulation is often observed in lymphomas, as in the case of follicular lymphoma. Though FISH revealed the absence of any chromosomal translocations involving *BCL2*, which maps on chromosome 18, we detected up-regulation of its expression level in treated cells, both at the mRNA (FIGURE 6a) and protein levels (FIGURE 7), which may be compatible with the acquirement of an anti-apoptotic capability by treated cells.

DISCUSSION

A potential transforming capability by *E. tirucalli* extracts has been suggested by a single publication in the last twenty years (27). No further studies have been performed since then to highlight through which molecular mechanisms it occurred. In this study, we report the results on cell proliferation and cell death, expression of EBV-antigens and induction of chromosomal abnormalities in LCL and cord-blood derived cell lines after treatment with *E. tirucalli* plant extracts. Results have been almost completely matching between the two cell lines, though the freshly established cord-blood derived cell line is more likely to represent the *in vivo* situation in respect with an LCL, as a prolonged *in vitro* culture in the latter could determine the accumulation of pre-existing genetic abnormalities.

E. tirucalli treatment determined a reduction of cell proliferation and a concomitant increase of cell death. This result is in contrast to what reported by literature for PBMCs treated with *E. tirucalli* (34), which seems to result in an increased proliferation rate following treatment. Quite interestingly, we were not able to reproduce these results neither in LCLs or cord blood-derived cells. One possible explanation could be that the increase of cell proliferation previously reported (34) has been observed mostly on the $CD3^+$ subpopulation within PBMC, which represents lymphoid T-cells, whereas our results are referred to EBV-infected B-cells.

Cell death was quite high in all the treated cells, being highest with the highest concentrations, and seemed to depend on necrosis due to the plant toxicity, rather than to apoptosis, as no DNA laddering nor caspase activation was detected in cells treated with *E. tirucalli* (Fig. 1c-d). The possibility that cell death was due to activation of the lytic cycle of EBV, as possibly suggested by Zebra expression, was ruled out by treating an EBV-negative cell line using the same experimental conditions. Cell death levels in this

cell line were comparable to those observed in EBV-positive cells, thus suggesting that cell death was due to plant toxicity.

On the contrary, treatment with *E. tirucalli* extracts modulated the expression of EBV-antigens. In particular, the expression of early antigens, and a marked up-regulation of LMP1 were observed after treatment. LMP1 expression may be relevant for NF-κB activation (35) and apoptosis (36). A similar pattern of expression of EBV antigens expression was observed in LCL and cord-blood cells, with the exception of gp350, which was not expressed in cord-blood after treatment, whereas a weak expression was observed in LCL.

Although it has been previously reported that cells treated with *E. tirucalli* and cultured for one year accumulated genetic abnormalities, here we report for the first time that as less as a five-day treatment of *E. tirucalli* was able to determine genomic abnormalities both in LCL and CB. In particular, in both cases polysomies were observed, being more evident the higher the concentration of *E. tirucalli* was. The possibility that the observed polysomies observed in LCL could be due to the accumulation of several genetic alterations, which may happen to cells cultured for many years, was ruled out by the observation that polysomies were induced by *E. tirucalli* treatment also in newly established CB cells, with a normal caryotype. In particular, polysomy of chromosome 8 was detected by FISH through specific centromeric probes, resulting in an increased number of copies of the *MYC* oncogene, as detected by FISH. Though Aya et al. reported the occurrence of specific chromosomal translocation in about 10% of treated cells after one year of culture, we did not detect any specific translocations. One possible explanation could be that our analyses have been performed after only five days of treatment, which were enough to induce polysomies, but may be not sufficient to let the chromosomal translocation occur. It is presumable that keeping these already genetically altered cells

growing for a longer period of time might determine the onset and the accumulation of further genetic alterations, as translocations. In any case, the increased number of *MYC* copies could mimic the effects of the *MYC* activation due to the translocation, as observed in most BL. It is worth noting, nevertheless, that BL cases lacking *MYC* translocation do exist, in which the *MYC* expression level is increased due to different mechanisms (4, 5, 37). Of interest, polysomies were detected only for chromosome 8, suggesting a predilection for a specific genetic locus alteration after *E. tirucalli* treatment.

Interestingly, EBV reactivation may be crucial as its proteins may induce the expression of cellular genes. In particular, EBNA2 may induce *MYC* expression (38), whereas LMP1 may induce *BCL2* expression (39). This may be of help to explain *BCL2* hyper-expression following *E. tirucalli* treatment, as no chromosomal translocations, neither genetic alterations involving *BCL2* have been detected. It is reasonable to hypothesize *BCL2* up-regulation may be due to LMP1, whose expression is induced by *E. tirucalli*. In addition, EBNA2 over-expression, which is itself induced by *E. tirucalli,* may also contribute to hyper-expression of *MYC*. The observation of multiple signals for chromosome 8 indicates an additional mechanism explaining *MYC* up-regulation, which can synergistically act with EBNA2-induced *MYC* expression, in determining higher expression levels of c-MYC. The over-expression of c-MYC should lead to both an increase of cell proliferation and cell death, as *MYC* has proliferative and pro-apoptotic effects, thus keeping balanced cell number. *E. tirucalli*-treated cells showed a marked up-regulation of the anti-apoptotic gene *BCL2*, though no genetic alterations for this gene had been detected by FISH. The up-regulation of *BCL2* may counteract the pro-apoptotic effect due to c-MYC over-expression and could give the treated cells a growth advantage, which may contribute to malignant transformation.

Collectively, our preliminary data suggest that *E. tirucalli* may cooperate in inducing malignant transformation, due to its modulation of the expression of the latency genes of EBV, and the up-regulation of two key factors as *BCL-2* and *MYC.* In particular, the over-expression of c-MYC seems to rely on the induction of polysomies after treatment, rather than chromosomal translocations.

These observations suggest that *Euphorbia tirucalli* extracts and, possibly, extracts of other plants, which are known to have similar EBV-activating properties, could act as cofactors for lymphomagenesis in endemic African Burkitt's lymphoma.

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TABLE 1: List of antibodies and their respective concentrations

Primary Antibody	Dilution	Company
EBNA-1	1:50	Novus Biologicals
EBNA-2	1:50	Dako
ZEBRA	1:50	Santacruz
LMP1	1:50	Dako
EA	1:50	Santacruz
Gp350	1:100	Santacruz
BCL6	1:30	Dako
BCL2	1:150	Dako
CD20	1:150	NeoMarkers
CD27	1:50	NeoMarkers
IgM	1:10000	Dako
CD30	1:50	NeoMarkers
CD10	1:20	NeoMarkers
CD79	1:50	NeoMarkers
IgD	1:50	NeoMarkers
Irf-4	1:50	Dako
CD138	1:100	Dako
Caspase 3	1:50	Abcam

TABLE 2: Immunocytochemistry (ICC) of EBV-encoded proteins in cells treated with *E. tirucalli* at the concentration of 10 μg/ml vs. untreated cells, expressed as percentage of positive cells out of total cells. Comparable results were obtained in LCL and cord blood-derived cells.

	Control (%)	***E. tirucalli* 10 μg/ml (%)**
Zebra	0	6
Ea-d	40	50
LMP1	50	90
Gp350	0	0
EBNA1	5	30
EBNA2	10	80

TABLE 3: FISH Analysis on *E. tirucalli* treated vs. untreated cells

	Control	***E. tirucalli* 10 μg/ml**
BCL2	No translocation	No translocation
BCL6	No translocation	No translocation
IgH (chromosome 14)	No translocation	No translocation
IgL (chromosome 22)	No translocation	No translocation
c-MYC (chromosome 8)	No translocation	Polysomies, no translocation

Key to figures:

Figure 1 (a-d): a) Cord blood-derived cells were treated with different concentration of *E. tirucalli* for five days, and proliferation has been monitored. Methanol-treated cells, with no *E. tirucalli* extract, have been used as a control. A dose-dependent decrease of proliferation rate is observed in *E. tirucalli*-treated cells ($p<0.05$). The graph is representative of three different experiments. Error bars represent standard deviation between duplicates. **b)** Cell cycle analysis by FACS on untreated (upper part) and *E. tirucalli*-treated cells (lower part). Tables indicate the percentage of cells in each cell cycle stages, where M1 indicates total number of dead cells (apoptotic and necrotic cells), M2 indicates G0/G1, M3 cells in S phase and M4 the G2/M phase. Treated cells show a higher number of the M1 fraction. **c)** Electrophoresis on agarose gel of untreated (lanes 1-2) and *E. tirucalli*-treated cells (lanes 3-4). No DNA laddering indicative of cell death by apoptosis is visible. **d)** ICC for caspase 3. No caspase activation is detected following *E. tirucalli* treatment.

Figure 2: To rule out the possibility that the decrease of cell proliferation and the concomitant increase in cell death observed in *E. tirucalli*-treated cells was due to induction of the lytic pathway of EBV, an EBV-negative cell line (Ramos) was cultured in the same experimental conditions as cord blood derived cells. *E. tirucalli* treatment had the same effects on cell proliferation and cell death, independently of EBV status. Treated cells showed a decreased cell proliferation ($p<0.05$). The graph is representative of three different experiments. Error bars represent standard deviation between duplicates

Figure 3: Immunocytochemistry of untreated (left) and *E. tirucalli*-treated cells (right). The expression of EBV-coded products was monitored.

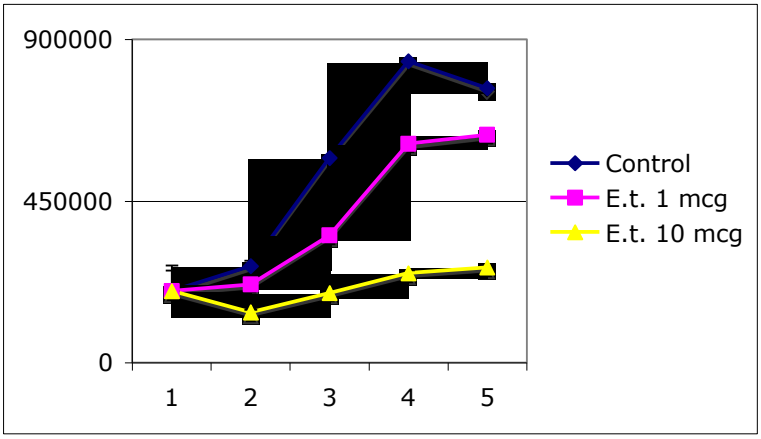
Figure 4: FISH analysis for *BCL2* (a, b) and *MYC* (c, d) for untreated (a, c) and *E. tirucalli*-treated cells (b, d). No balanced translocations have been detected, though multiple signals for *MYC* have been identified. Arrows indicate cells with multiple *MYC* signals.

Figure 5: To assess whether multiple *MYC* signals relied on *MYC* gene amplification or on chromosomal 8 polyploidy, a centromeric probe for chromosome 8 was used. Our results indicated the presence of multiple signals for chromosome 8 in cells treated with *E. tirucalli*, consistent with a polysomy of chromosome 8. Arrows indicate cells with multiple chromosome 8 and *MYC* signals.

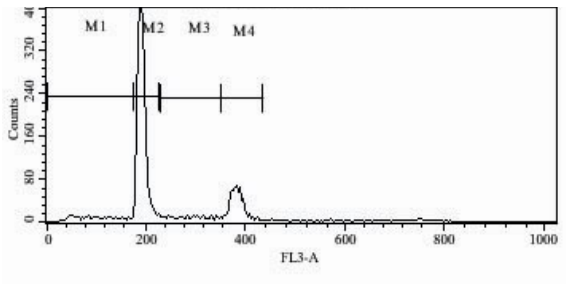
Figure 6 (a-b): a) qRT-PCR for *BCL2* and *MYC* in untreated and *E. tirucalli*-treated cells. A marked up-regulation of both genes is observed following treatment. The graph is representative of three different qRT-PCR experiments. Error bars represent standard deviation between duplicates. **b)** Immunofluorescence of untreated (upper panel) or *E. tirucalli*-treated cells (lower panel). C-MYC expression increases following treatment. Magnification 40x.

Figure 7: ICC for *BCL2* in untreated (a) and *E. tirucalli*-treated cells (b). Magnification 40x.

Figure 1

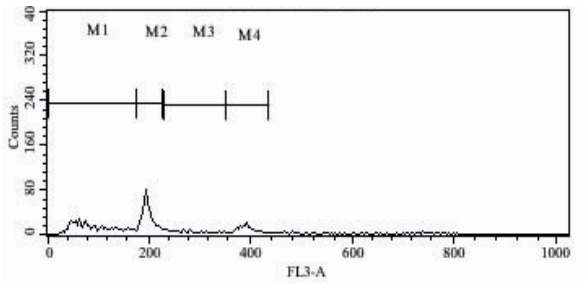


a



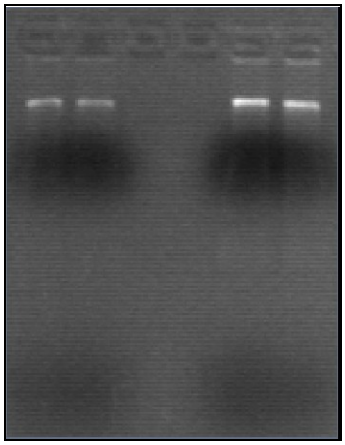
Untreated cells	%
M1	6.65
M2	66.82
M3	6.24
M4	17.41

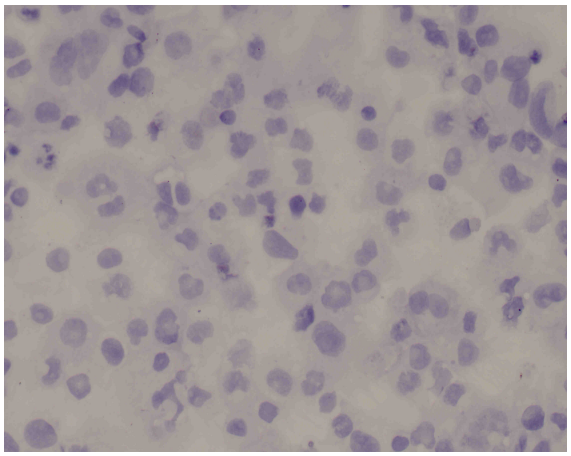
Treated cells (10 µg/ml)	%
M1	37.78
M2	37.12
M3	8.80
M4	12.19



b

1 2 3 4





1-2: Untreated cells

3-4: Treated cells

c d

Figure 2

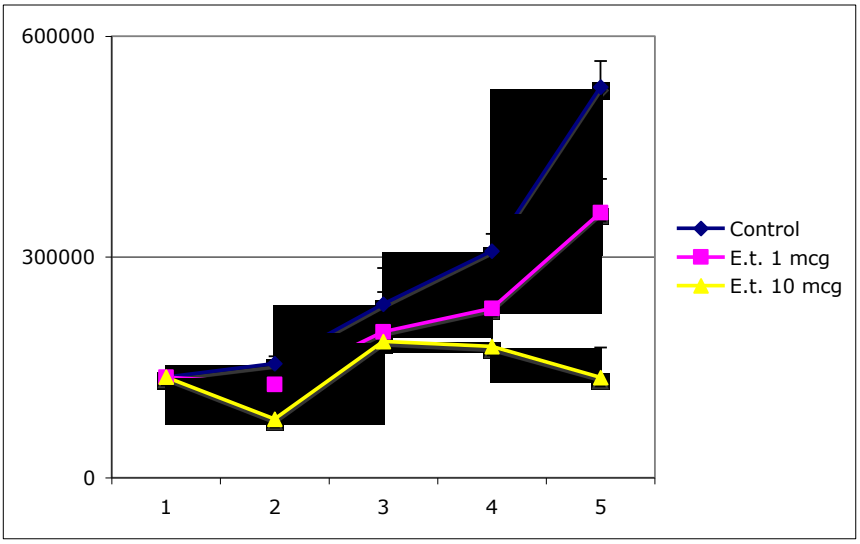
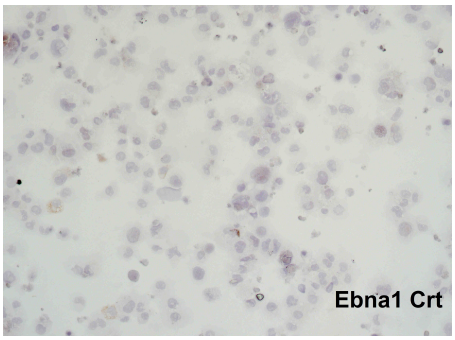


Figure 3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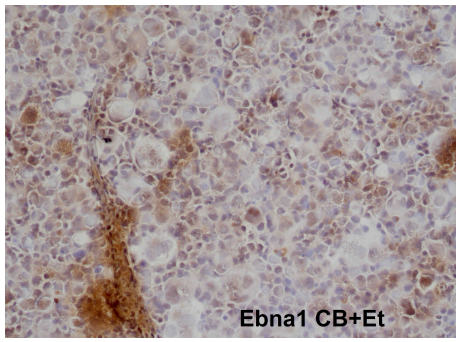
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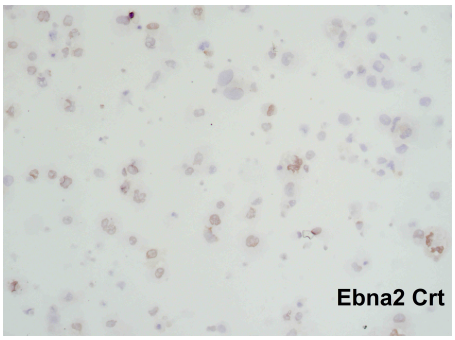
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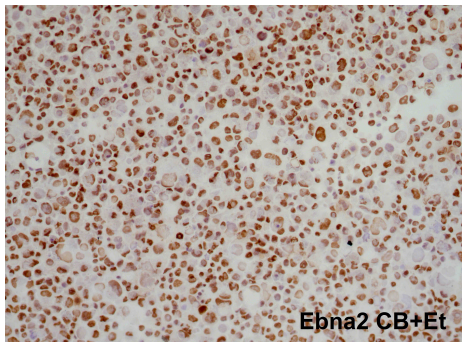
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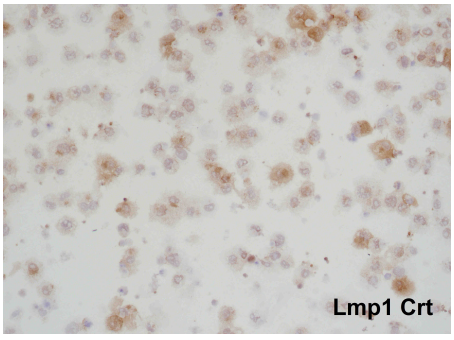
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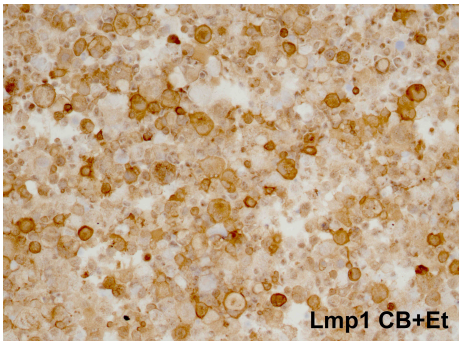
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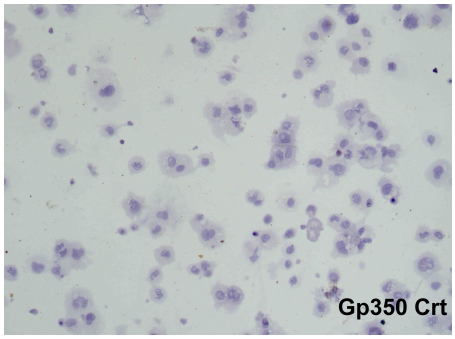
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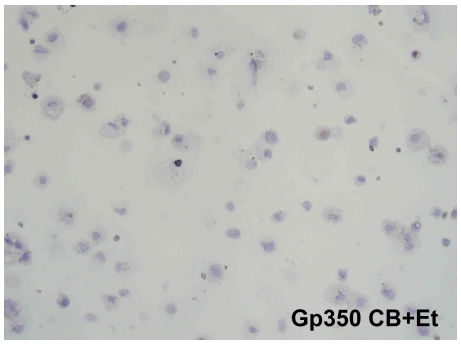
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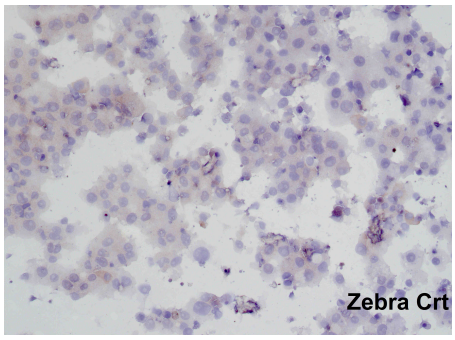
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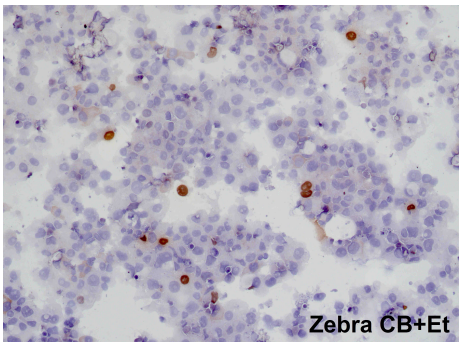
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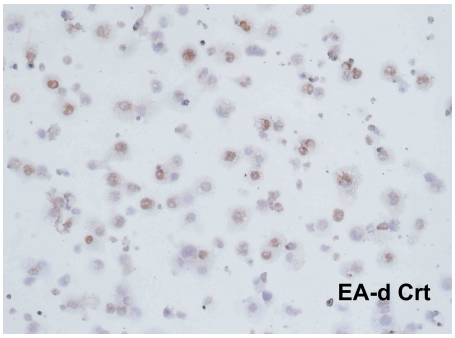
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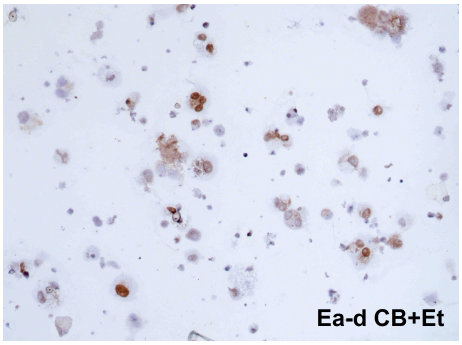
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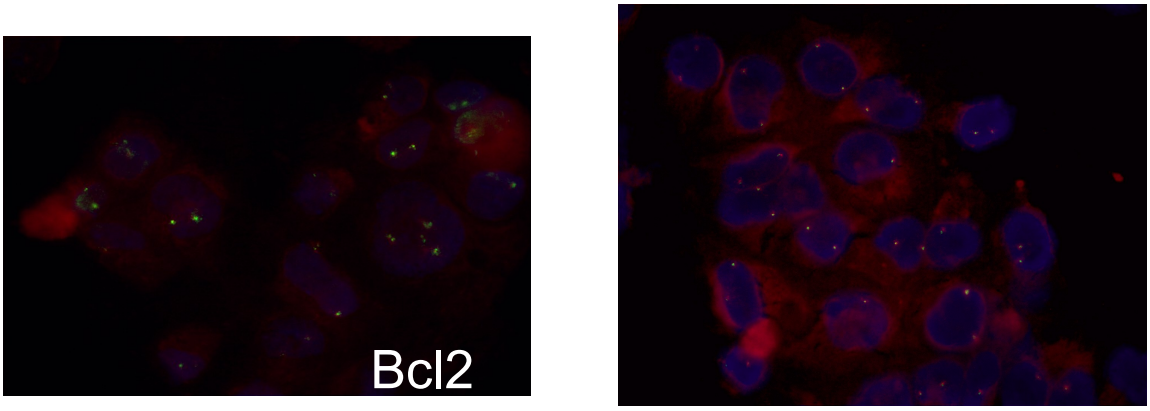
Figure 4



a b

c d

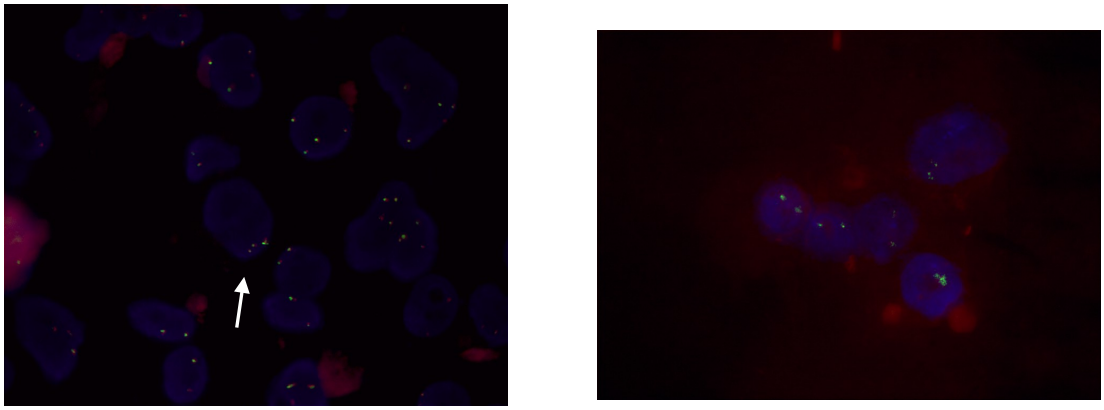


Figure 5

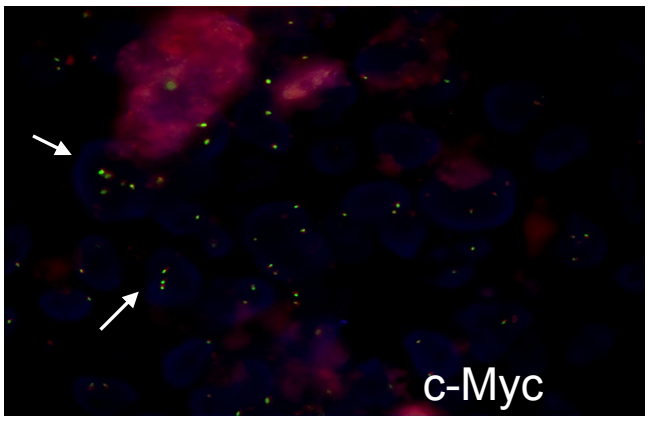
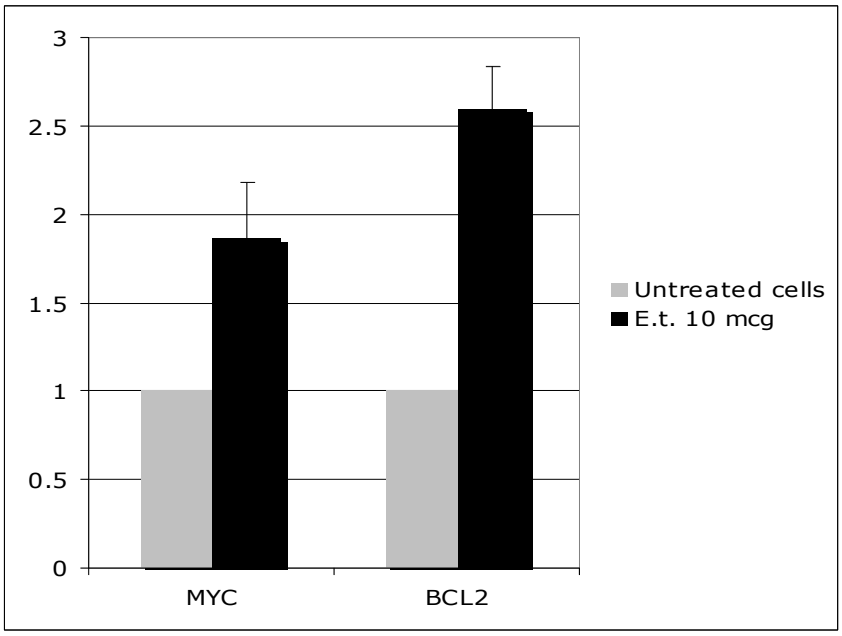
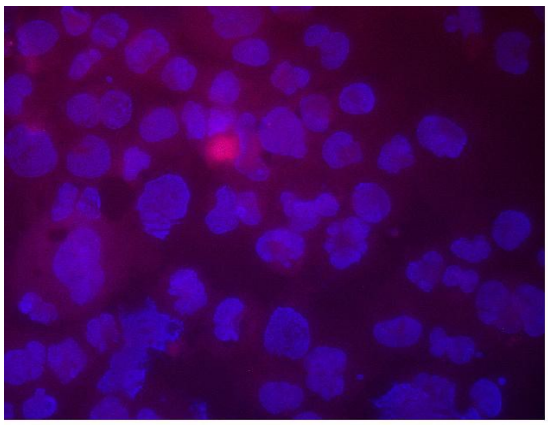


Figure 6





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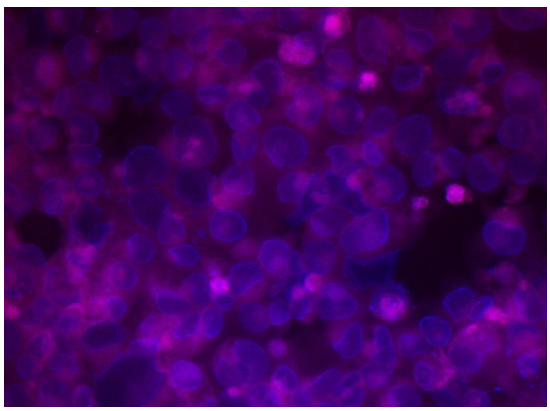

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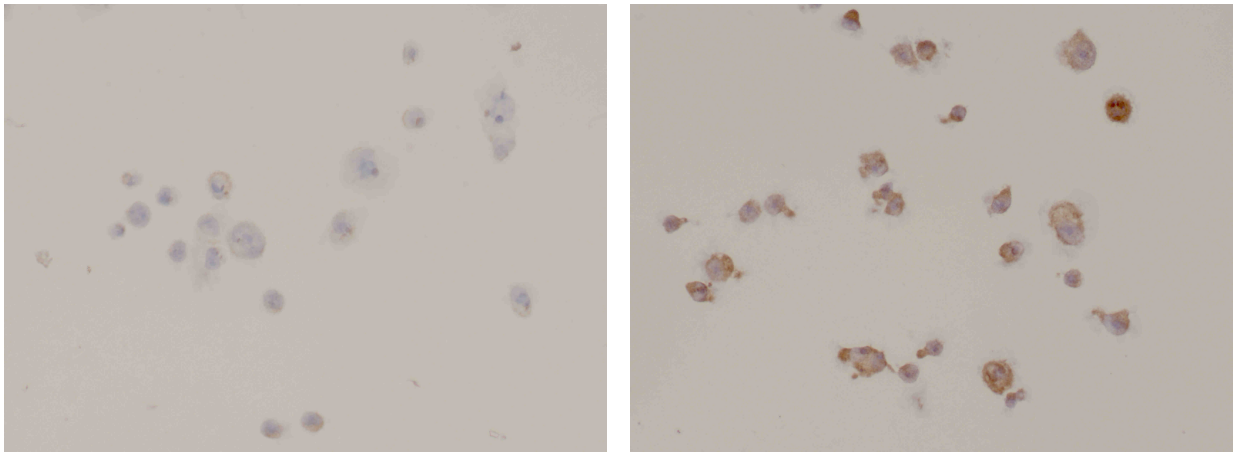
Figure 7



a b

The Alteration of Lipid Metabolism in Burkitt Lymphoma Identifies a Novel Marker: Adipophilin

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Ann M. Moormann, Editor

Abstract

Background

Recent evidence suggests that lipid pathway is altered in many human tumours. In Burkitt lymphoma this is reflected by the presence of lipid droplets which are visible in the cytoplasm of neoplastic cells in cytological preparations. These vacuoles are not identifiable in biopsy section as lipids are "lost" during tissue processing.

Methods and Results

In this study we investigated the expression of genes involved in lipid metabolism, at both RNA and protein level in Burkitt lymphoma and in other B-cell aggressive lymphoma cases. Gene expression profile indicated a significant over-expression of the adipophilin gene and marked up-regulation of other genes involved in lipid metabolism in Burkitt lymphoma. These findings were confirmed by immunohistochemistry on a series od additional histological samples: 45 out of 47 BL cases showed strong adipophilin expression, while only 3 cases of the 33 of the not-Burkitt lymphoma category showed weak adipophilin expression ($p<0.05$).

Conclusions

Our preliminary results suggest that lipid metabolism is altered in BL, and this leads to the accumulation of lipid vacuoles. These vacuoles may be specifically recognized by a monoclonal antibody against adipophilin, which may therefore be a useful marker for Burkitt lymphoma because of its peculiar expression pattern. Moreover this peptide might represent an interesting candidate for interventional strategies.

Introduction

Burkitt lymphoma (BL) is listed in the World Health Organization (WHO) classification of tumours of haematopoietic and lymphoid tissues as an 'aggressive B-cell non-Hodgkin lymphoma'(B-NHL) [1]. Based on epidemiological features, BL is subdivided into three variants: endemic, sporadic and immunodeficiency-associated. While the Epstein–Barr virus (EBV) is associated with 98% of the endemic BL, it is seen only in 20% of sporadic cases, and 30–40% of immunodeficiency-associated cases [2]. Histologically BL is characterized by a monotonous infiltrate of medium-sized blastic lymphoid cells that show round nuclei with clumped chromatin and multiple nucleoli and by the presence of a 'starry sky' pattern. On fine needle aspiration cytology (FNAC), BL shows very typical lipidic vacuoles in the cytoplasm of lymphoid cells which represents a diagnostic hallmark [3]. Unfortunately, these vacuoles cannot be seen on histological preparations because during waxing and fixation they are largely lost from tissues, and a combination of several diagnostic techniques (such as morphology, immunophenotyping or genetic analysis) is necessary to achieve the diagnosis of BL [1]. The tumour cells are positive for CD79a, CD20, CD10, BCL6, CD38, and are negative for BCL2, Mum-1, CD44 and CD138. The proliferation fraction measured by Ki-67 is nearly 100% [2]. At molecular level, BL is characterized by the chromosomal rearrangement of MYC, in the form of reciprocal translocation juxtaposing the MYC gene at 8q24, to the immunoglobulin heavy chain (IGH) locus at 14q32, or the IGK (2p11) or IGL (22q11) light chain loci [2].

The presence of lipid vacuoles in the cytoplasm of BL cells on FNAC may suggest that biosynthesis of lipids and other macromolecules is altered in BL. Previous study has found that lipogenic pathway is activated in some tumours (i.e. hepatocellular carcinoma, clear cell renal carcinoma, adenocarcinoma of the colon, sebaceous tumours) [4]–[5]. In

contrast to normal cells, neoplastic cells rely mainly on anaerobic glycolysis, a phenomenon known as Warburg effect [5]. To sustain the rapid proliferation and to counteract the hostile environment, cells must increase the rate of metabolic reactions to provide adenosine triphosphate, lipids, nucleotides and amino acids necessary for daughter cell production [6]–[7]. A recent paper on lipid metabolism in B-NHL has shown dysregulation of fatty acid synthesis and increase of glycolysis in these tumours, suggesting fatty acid synthase enzyme as a candidate for molecular target therapy [8].

The present study was designed to analyse the lipid metabolism in BL and to identify a novel possible marker. We investigated by gene expression profile (GEP) the genes involved in the lipogenic pathway in 13 BL and 20 diffuse large B-cell lymphoma (DLBCL) cases. We observed differences in lipid metabolism between BL and DLBCL and identified adipophilin (adipocyte-differentiation-related protein) as the only member of the Perilipin, Adipholin, Tail-interacting protein of 47 kDa (TIP47) (PAT)-proteins family strongly expressed in BL [9]. The GEP results were validated on a series of additional cases classified according to the recent algorithm proposed by Naresh et al. [10] by immunohistochemistry, using a monoclonal antibody against the adipophilin to confirm lipid accumulation in standard formalin-fixed paraffin-embedded samples [4]. We observed that 45 out of 47 cases of BL showed positivity for adipophilin whereas only 3 out of 33 cases of the not-BL category were adipophilin-positive. These results suggest adipophilin as a novel marker for BL.

Materials and Methods

Ethics Statement

Ethics approval for this study was obtained from the Institutional Review Board at the University of Siena (Italy) and from the Ethics and Research Committee at the Lacor Hospital (Uganda). Informed written consent was obtained in all cases.

Case Selection

A total of sixty formalin-fixed and paraffin-embedded specimens were investigated at the Department of Human Pathology and Oncology, Anatomical Pathologic Section, University of Siena, Italy; the cases had been accrued locally and from Lacor Hospital, Uganda. The initial diagnosis of these cases was: BL in 43, diffuse large B-cell lymphoma (DLBCL) in 30 and B-cell lymphoma unclassifiable with features intermediate between DLBCL and BL (DLBCL/BL) in 7 [11].

Histological and Immunohistochemical Studies

The slides were reviewed by two expert haematopathologists (LL, SL) and classified according to the scoring system recently designed by Naresh et al. [10] for aggressive B-cell lymphomas which distinguishes BL and not-BL. In fact, from a practical standpoint it is more beneficial to focus on two categories, given that the prognosis for the DLBCL/BL is uniformly poor [10]. According to the algorithm, we identified 47 BL and 33 not-BL cases (Figure 1). Histological sections (4-µm thick) were placed on positively charged glass slides (ProbeOn Plus; Fisher Scientific, Pittsburgh, PA, USA). The staining was performed on Bond Max automated immunostainer (Leica Microsystem, Bannockburn, IL, USA), with adipophilin antibody (pre-dilute AP 125; ProgenBiotechnik GmbH Maabstrasse Heidelberg, Germany) with controls in parallel. No epitope retrieval was used. Ultravision Detection System using anti-Polyvalent HRP (LabVision, Fremont, CA, USA) and diaminobenzidine (DAB, Dako, Milan-Italy) as chromogen was used. The

pattern of immunostaining as well as the labelling intensity was evaluated independently in each of the 80 cases. The statistical association between the distribution of expression of adipophilin and the diagnostic category (BL and not-BL) was analysed using $\chi 2$–test and Fisher's exact test, with $P<0.05$ considered as being statistically significant.

Gene Expression Analysis

We analyzed GEP data of 13 BL and 20 DLBCL (10 GCB-type and 10 ABC-type), previously generated by using the Affymetrix HG-U133 2.0 plus microarray (Affymetrix, Inc. http://www.affymetrix.com/support/index.affx) and available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26673 (GSE26673). For technical details, see reference 12 [12]. In particular, we focused on the expression of ADFP (PLIN2), identified by a specific probe set in the HG-U133 2.0 plus GeneChip (209122_at). In addition, we studied the expression of genes whose activity is known to be related to lipid metabolism, such as, SCD, SCD5, FASN, USF1, PPARA, and represented in the microarray by the following probe sets: 200831_s_at, 211708_s_at, 220232_at, 212218_s_at, 231768_at, 223437_at, 223438_s_at, 226978_at. Further details on patients as well as on GEP generation were previously reported [9]. Supervised analysis and hierarchical clustering were performed as previously described by using GeneSpring GX11.0 platform (Agilent, USA) [9.12]. Additional statistical analyses were carried on with the StatView 5.0 software package (SAS Institute Inc, Cary, NC). Anova, unpaired T-Test and when required (specifically, when sample size was inferior to 10 cases in at least 1 group) a non-parametric Mann-Whitney (MW) were adopted for GEP data analyses and, in particular, for comparing ADFP expression in different subgroups. The limit of significance for all analyses was defined as $P<0.05$; two-sided tests were used in all calculations.

Results

ADFP (PLIN2) Gene is More Expressed in Burkitt Lymphoma than in DIFFUSE Large B-cell Lymphoma

A previous GEP study on BL cases revealed that adipophilin is the only member of the PAT-proteins family expressed in BL [9]. Using the same set of data, we measured the expression of ADFP in BL and DLBCLs and found a significant over-expression in the former. Specifically, BL presented significant higher level than both germinal center (GCB)-DLBCL ($p=0.04$) and activated B-cell (ABC)-DLBCL ($p=0.03$), while no difference was recorded among the DLBCL subtypes (Figure 2). Moreover, supervised analysis revealed that 5 other genes involved in lipid metabolism were differentially expressed (fold change >2; $p<0.05$) in BL and DLBCL. While FASN, SCD5 and USF1 were up-regulated in BL, SCD and PPARA were up-regulated in DLBCLs. (Figure 3; Table 1).

Adipophilin is Strongly Expressed in Burkitt Lymphoma Cases

To confirm GEP results, adipophilin expression was investigated by immunohistochemistry in BL and not-BL cases, classified according to the recent algorithm proposed by Naresh et al. [10]. Distinct patterns of adipophilin expression, highlighting lipid vacuoles, were observed among the two different categories of lymphoma. A strong immunoreactivity, characterized by single or multiple droplets in the cytoplasm and clustering of these to the outer nuclear membrane, was observed in 45 out of 47 BL cases (Figure 4A-B). In these cases, smaller lipid droplets were also present. Weak positivity characterized by dispersed fine lipid droplets in the cytoplasm of a small minority of cells was detected in 3 out of 33 cases of the not-BL category (Figure 4C-D). 30 out of 33 not-BL cases did not show any expression of adipophilin (Figure 4E-F). Macrophages showed a granular staining within the cytoplasm, and this served as an

internal positive control. The evaluation of immunohistochemical expression of adipophilin is summarized in table 2. The proportion of cases positive for adipophilin expression was significantly higher ($p<0.05$) in BL cases than in the not-BL cases.

Interestingly, the three cases of the not-BL category that showed weak, fine positivity were characterized by a diffuse proliferation of medium- to large-sized cells with irregular nuclear contours and relatively large nucleoli. Starry-sky macrophages, mitoses and apoptosis were prominent, and reactive small lymphocytes were scanty. This morphological features corresponded to score 1 (range: 0–3) on the algorithm for aggressive B-cell lymphomas proposed by Naresh et al. [10] and may well represent B cell lymphoma, unclassifiable with features intermediate between DLBCL and BL [11].

Discussion

BL is an aggressive B-cell lymphoma with a worldwide distribution. None of the histological, immunohistochemical or molecular parameters can be singly used for the diagnosis of BL and the WHO classification suggests that a combination of several techniques is necessary [1]. BL is a potentially curable malignancy if correctly diagnosed, even in the resource-poor settings where BL is the most common malignancy in paediatric patients [2]. In these settings, often, performing biopsies is not feasible and the diagnosis of BL is made on FNAC specimens by identifying the cytoplasmic lipid vacuoles within the lymphoid cells. However, FNAC is not always adequate for accurate diagnosis and cytological specimens do not support further investigations [3]. In this study, we have attempted to find a novel marker that can detect lipid vacuoles in histological samples by analysing the lipid metabolism in BL at both gene and protein level.

GEP showed that FASN, SCD5, USF1 are up-regulated in BL whereas SCD and PPRA are down-regulated. In addition, we identified the adipophilin as the only member of PAT-

proteins family to be significantly over-expressed in BL [9]. PAT-proteins is a family of lipid droplet-associated proteins which also includes adipophilin, perilipin and TIP47. These proteins are involved in the formation, maintenance, modification and involution of lipid droplets [13]–[14]. While the expression of perilipin is thought to be restricted to adipocytes and certain steroidogenic cells, adipophilin and TIP47 are nearly ubiquitously expressed [15]. In particular, adipophilin is localized to the surface of lipid droplets [4] and is mainly involved in fatty acids transport and in preserving the cellular triacylglycerols content [4], [16]–[17]. Tumour cells require de novo synthesis of lipids for membrane assembly and biosynthesis of other macromolecules (such as proteins and nucleic acids) to rapidly dividing [18]. Newly generated fatty acids are promptly incorporated into membrane lipids and triacylglycerol stores to accommodate the dramatic proliferative rates of some types of neoplastic cells [19]. Adipophilin over-expression in BL may thus reflect the up-regulation of lipogenic pathway and might be related to the high proliferation rate of this tumour, the fastest growing tumour in humans.

The results generated by GEP, were validated at protein level by immunohistochemistry using an antibody against adipophilin. BL was characterized by strong immunoreactivity and by the presence of single or multiple droplets in the cytoplasm and clustering of these droplets to the outer nuclear membrane whereas DLBCL showed no expression of adipophilin. A weak labelling may be observed in not-BL cases with features intermediate between DLBCL and BL. Macrophages showed a granular staining in the cytoplasm and this may explain the partial overlap between BL and DLBCL observed at RNA level. These findings suggest adipophilin as a novel marker that can be useful for the diagnosis of BL in histological sections and may be a reliable marker in challenging cases such as DLBCL/BL.

In this paper we gave evidence, for the first time, that lipid metabolism is altered in BL. The extent to which metabolism plays a role in lymphomagenesis should not be underestimated. The change in metabolism cannot be purely attributed to alteration in allosteric and product/substrate regulation of the metabolic enzymes [20]–[24]. Metabolic reprogramming in neoplastic cells involves numerous genes (i.e. MYC, P53, H1F1, PTEN) that finally enhances anaerobic glycolysis, lactate production, and biosynthesis of lipids and other macromolecules [19]. The genes that regulate these metabolic pathways may thus serve as targets for specific therapies [23] and, among these, adipophilin may be an interesting candidate for interventional strategies (e.g. target therapy or vaccine therapy). A preliminary study has, in fact, investigated the possible use of adipophilin as a T-cell epitope to induce antigen-specific cytotoxic T-lymphocytes and mediate tumour cell lysis [25].

A better knowledge of lipid metabolism alteration in BL can potentially provide new markers to improve diagnosis and prognosis as well as novel therapeutic approaches for BL treatment [26].

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Key to figures:

Figure 1. Classification of cases according to the algorithm proposed by Naresh et al. After the revision of 43 BL, 30 DLBCL and 7 DLBCL/BL cases, 47 BL and 33 not-BL cases were identified.

Figure 2. ADPF gene over-expression in BL compared to DLBCL. Box plots represent the values distribution across the three considered categories (A); the extremities of the boxes indicate the first and third quartile. The horizontal lines inside the boxes indicate median values; outlier values are indicated by blue dots. Histograms represent the mean expression values in the three groups (B).

Figure 3. Over-expression of genes involved in lipid metabolism in BL. SCD5, FASN and USF1 are up-regulated in BL; SCD and PPARA are upregulated in DLBCLs.

Figure 4. Adipophilin immunostain in BL and not-BL cases. (A) BL is characterized by medium-sized cells with a monotonous cohesive pattern of growth, round nuclei with finely clumped and dispersed chromatin, a high proliferation rate and ‘‘starry-sky’’ appearance [Haematoxilin-eosin (H&E), original magnification (O.M.)6200). (B) Neoplastic cells show strong positivity to adipophilin with single or multiple droplets in the cytoplasm, sometimes clustering the outer nuclear membrane (inset); the internal positive control is represented by macrophages which show granular positivity in the cytoplasm (arrows) (Adipophilin stain, O.M.6200; inset, O.M.6400). (C) An aggressive B-cell lymphoma with diffuse proliferation of medium- to large-sized cells with irregular nuclear contours and relatively large nucleoli corresponding to morphological score 1 according to the Naresh et al. scoring system is shown. Few small lymphocytes and starry-sky macrophages are also present (H&E, O.M.6200). (D) Adipophilin immunostain on the case depicted in figure C with neoplastic cells showing weak positivity characterized by singly scattered fine lipid droplets in the cytoplasm (Adipophilin stain, O.M.6200). (E)

Morphological features of a diffuse large B-cell lymphoma (H&E, O.M.6200). (F) Adipophilin immunostain on the case depicted in figure E is entirely negative (Adipophilin stain, O.M.6200).

Table 1. Genes involved in lipid metabolism in BL other than PLIN2.

Probe Set ID	p-value	Unigene (Avadis)	Gene Symbol	Gene Title	Regulation	Entrez Gene
200831_s_at	0,006725255	**Hs.558396**	SCD	stearoyl-CoA desaturase(delta-9-desaturase)	down	6319
211708_s_at	9,94E-04	**Hs.558396**	SCD	stearoyl-CoA desaturase(delta-9-desaturase)	down	6319
212218_s_at	0,033671293	**Hs.83190**	FASN	fatty acid synthase	up	2194
220232_at	0,009198004	**Hs.379191**	SCD5	stearoyl-CoA desaturase 5	up	79966
223437_at	3,85E-06	**Hs.103110**	PPARA	peroxisome proliferator-activatedreceptor alpha	down	5465
223438_s_at	1,79E-05	**Hs.103110**	PPARA	peroxisome proliferator-activatedreceptor alpha	down	5465
226978_at	3,51E-07	**Hs.103110**	PPARA	peroxisome proliferator-activatedreceptor alpha	down	5465
231768_at	0,001676206	**Hs.414880**	USF1	upstream transcription factor 1	up	7391

Table 2. Adipophilin expression, including intensity of staining and pattern of expression.

Adipophilin expression, including intensity of staining and pattern of expression.

Histotype	N. positive (%)	N. negative (%)	Intensity of staining	Pattern of expression
BL	45 (96%)	2 (4%)	strong	single or multiple droplets into the cytoplasm, clusteringaround the outer nuclear membrane
not-BL	3 (9%)	30 (91%)	weak	single, very small droplets into the cytoplasm

BL: Burkitt lymphoma; N.: number of cases; %: percentage.

Figure 1

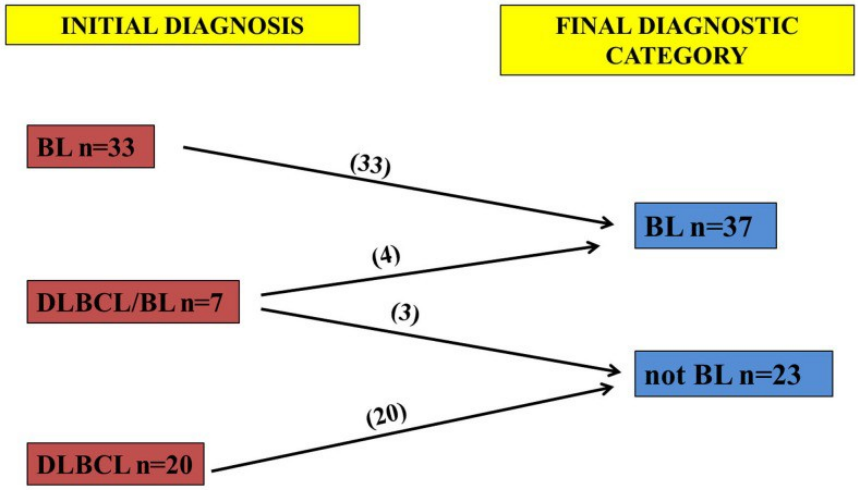
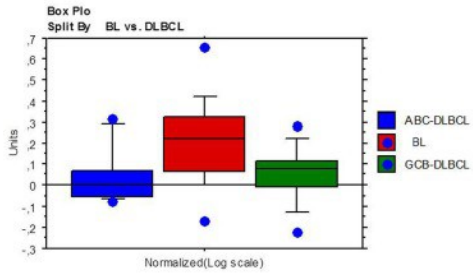
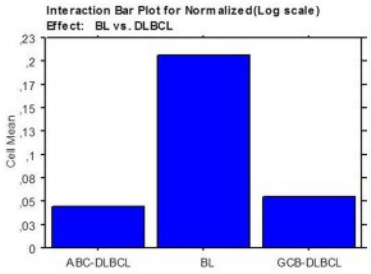


Figure 2





Fisher's PLSD for Normalized(Log scale)
Effect: BL vs. DLBCL
Significance Level: 5 %

	Mean Diff.	Crit. Diff	P-Value	
ABC-DLBCL, BL	-,161	,143	,0280	S
ABC-DLBCL, GCB-DLBCL	-,010	,152	,8916	
BL, GCB-DLBCL	,151	,143	,0386	S

Figure 3

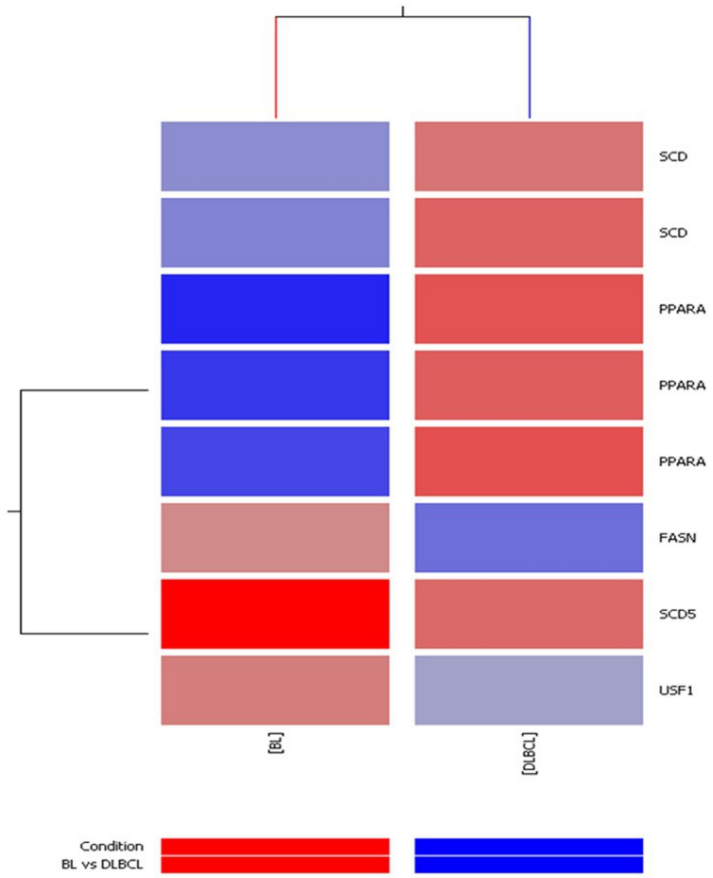
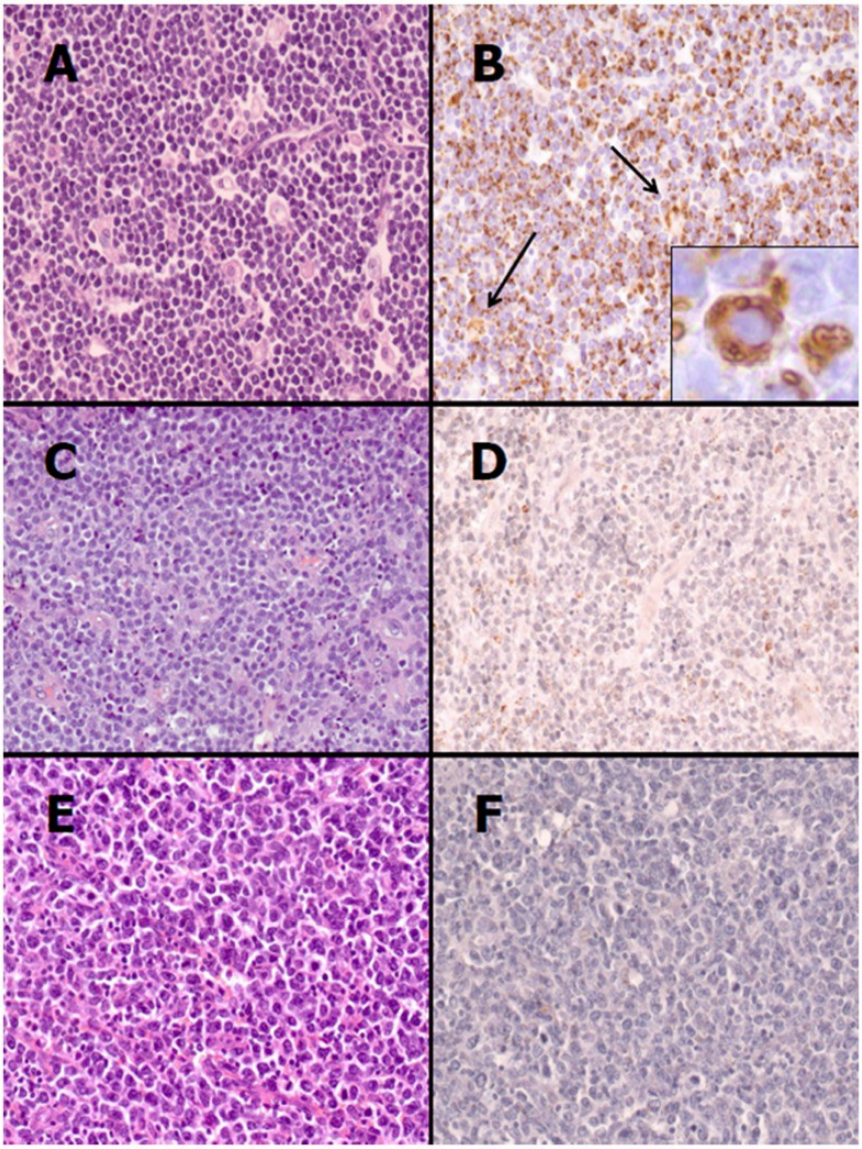

SCD
SCD
PPARA
PPARA
PPARA
FASN
SCD5
USF1
[BL]
[DLBCL]
Condition
BL vs DLBCL

Figure 4



Conclusions

Unravelling the molecular bases of cancer has been matter of interest for the past few decades and the advent of more sophisticated molecular techniques has offered an additional level of knowledge that completes the information obtained by a more traditional approach. Despite the undisputed importance of assessing grading and staging of each tumor, understanding its biological uniqueness helps a more detailed and subtle classification and the design of more personalized therapeutic protocols. The understanding that cancer should be considered a genetic disease has triggered a high number of research studies aimed at the identification of novel biological markers, which could be used in combination with more traditional ones, and has led to a more precise classification of tumors, in which sub-types can be identified based on molecular profiles. On the other hand, it would be dismissive not to consider that cancer is a very complex disease, in which genetic alterations may be triggered and/or supported by the existence of particular environmental conditions and the presence of specific pathogens. A viral etiology of cancer has now been acknowledged for about 20-30% of all tumors and this estimate will probably surge in the next few years. It should be also kept in mind that trying to unravel cancer is crucial not only to understand the etiology of the disease, but especially to improve prognosis of patients and guarantee a better quality of life. Therefore, a more comprehensive approach should be adopted when dealing with cancer patients, which takes into account all of these aspects with the aim of offering a more successful and personalised therapeutic regimen.