**Low Rap1-interacting factor 1 and sirtuin 6 expression predict poor outcome in radiotherapy-treated Hodgkin's lymphoma patients**

Running title: Sirtuins, Rif1 in Hodgkin's lymphoma

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**Abstract**

Sirtuins (SIRTs) are a family of histone deacetylases which widely regulate cellular metabolism and are also involved in DNA repair. Rap1-interacting factor 1 (Rif1) and $O^6$-alkylguanine DNA alkyltransferase (MGMT) are DNA-repair enzymes which may potentially be involved in resistance to treatment of classical Hodgkin's lymphoma (HL).

We assessed the expression levels of (previously unstudied) SIRT1, SIRT4, SIRT6, Rif1 and MGMT immunohistochemically in 85 patients with untreated classical HL.

Aberrant distributions of SIRT1, SIRT4 and SIRT6 were detected in Hodgkin's neoplastic Reed–Sternberg (RS) cells compared with reactive elements. Low-level expression of both Rif1 and SIRT6 predicted dismal relapse-free survival in radiotherapy-treated patients (multivariate analysis; HR 8.521; 95% CI 1.714–42.358; p=0.0088).

Expression levels of SIRT1, 4 and 6 were abnormally distributed in RS cells, suggesting a putative role of aberrant acetylation in classical HL carcinogenesis. Rif1 and SIRT6 may also have substantial prognostic and even predictive roles in classical HL.

## Introduction

Hodgkin's lymphoma (HL) is histologically an atypical cancer, because it consists of a few neoplastic Reed–Sternberg (RS) cells and mostly reactive cellular infiltrate [1]. RS cells originate from B cells, but B-cell-type immunoglobulin expression is nonexistent. The reactive cellular infiltrate consists mainly of immune B and T cells, fibroblasts, specialized mesenchymal stromal cells and endothelial cells. The infiltrate helps RS cells survive via several forms of interaction [2].

Modern treatments can result in cure in over 80% of all HL patients. However, HL survivors still have a reduced overall survival rate because of the increased incidence of cardiovascular diseases and secondary malignancies [3]. Therefore, it is vital to strike a balance between treatment efficacy and treatment-related toxicity [4]. Risk-factor scores are in wide clinical use for patients with limited-stage (IA–IIA) HL, and International Prognostic Scores (IPSs) are used for patients with advanced-stage (IIB–IV) disease, but prognosis still varies considerably within these classes [5].

When double-strand breaks (DSBs) occur in DNA, there are two main pathways to repair the damage: homologous recombination (HR) and non-homologous DNA end-joining (NHEJ). Rap1-interacting factor 1 (Rif1) is a highly conserved enzyme which is an integral component of NHEJ in DNA repair, primarily through repressing transcription and its recruitment by phosphorylated 53BP1 [6]. $O^6$-alkylguanine DNA

alkyltransferase (MGMT) is a crucial enzyme in genome stability, acting by repairing alkylated DNA adducts [7]. Thus, MGMT and Rif1 both reduce double-strand breaks in carcinoma cells, and expression levels of MGMT and Rif1 appear to have a significant role in the chemoresistance of solid tumors [8,9], but data concerning lymphomas is still very scarce.

In mammals, there are seven different silent information regulator 2 (SIRT, known also as sirtuins) isoforms (SIRT1–SIRT7). The sirtuin family is a highly conserved enzyme group catalyzing deacetylation and ADP-ribosylation [10]. SIRT1 is located in both the nucleus and cytoplasm, while SIRT4 is found in mitochondria and SIRT6 is located mostly in the nucleus [11]. Sirtuins also have diverse biological functions, being involved in cell division, differentiation, metabolism and survival [12]. SIRT1, SIRT4 and SIRT6 regulate cell metabolism as tumor suppressors, especially SIRT6, which is a key regulator of metabolism, controlling glucose homoeostasis. SIRT6 inhibits aerobic glycolysis, also known as the Warburg effect [13]. SIRT6 also attenuates HIF1α and Myc transcription by deacetylating H3K9 [14]. SIRT1 and SIRT6 promote genomic stability by inducing repair mechanisms for single- and double-strand breaks.

SIRT1 and SIRT6 are recruited to the DSB repair system, playing key roles in HR and NHEJ repair pathways. SIRT1 controls many significant HR repair proteins, such as Rad51 and NBS1 [15, 16]. There is also evidence that in post-mitotic neurons SIRT1 is involved in the regulation of NHEJ via ATM and HDAC1 proteins [17]. According to the current literature, SIRT4 is not involved directly in DNA damage

response (DDR). However, it regulates mitochondrial glutamine metabolism and is consequently involved in DDR. Loss of SIRT4 may also lead to increased genomic instability [18]. SIRT6 is involved NHEJ repairing system by stabilizing DNA-PK (DNA-dependent protein kinase), which promotes DBS to the NHEJ repair pathway [19]. Under oxidative stress, SIRT6 activates PARP-1 by stimulating its mono- and poly-ADP-ribosylase activity, this causes enhanced NHEJ and HR pairing pathway activation. [20]

SIRT1 overexpression has been observed in certain human cancers, including hepatocellular and colon carcinomas and diffuse large B-cell lymphoma [21–23], and it has also been connected to chemoresistance in breast and prostate cancer and myelogenous leukemia [24–26]. On the other hand, SIRT6 has mainly tumor-suppressive properties in pancreatic, skin, breast and prostate carcinomas [27–30], but it has also been linked to chemoresistance in breast and lung cancer [29,31]. Low SIRT4 expression has been reported in cases of leukemia, gastric cancer and bladder cancer [32–34].

Radiation therapy is an essential treatment modality in cases of HL. Ionizing radiation directly damages DNA, and therefore the overall DDR, including responses to DSBs, is a determinant of the response to radiotherapy. Mutations in genes that regulate DSBs, such as *BRCA1*, are associated with increased radiation sensitivity [35]. Also, immoderate activation of the DSB repair system contributes to tumorigenesis, chemo- and radioresistance and posttreatment relapse [36]. In hepatocellular carcinoma, PARP-1 inhibitors combined with radiotherapy have shown radiosentization effects

[37]. A clinical trial is currently being carried out to investigate the PARP inhibitor olaparib as regards enhancement of radiotherapy effects in cases of head and neck squamous cell carcinoma [38].

This study was undertaken to examine the unexplored roles of sirtuins 1, 4 and 6 in cases of HL, with special emphasis on associations between their expression, and therapy resistance or patient survival. Since the DNA-repair enzymes MGMT and Rif1 reflect activation of different DNA-repair mechanisms and have potential interactions with sirtuins, their expressions were also assessed.

## Materials and methods

### Patient material

The material consisted of 85 patients with histologically confirmed classical HL before the initiation of any treatments. All patients were treated with doxorubicin-bleomycin-vinblastine-dacarbazine (ABVD) chemotherapy in a first-line setting. Sixty-five patients also received involved-field radiotherapy after chemotherapy. Table 1 shows the demographics of the patients more precisely. All lymphomas were diagnosed and treated in Finland in 1997–2012. Forty-one patients were diagnosed and treated at Oulu University Hospital and 40 patients at Kuopio University Hospital. Four patients were diagnosed and treated at the Central Hospitals of Kajaani, Kemi, and Rovaniemi. Diagnoses were reviewed by a specialist hematopathologist. Accurate and updated patient information was gathered in each case from the hospital records.

Limited-stage risk factors included bulky mediastinal mass, elevated sedimentation rate, involved nodal regions ≥ 4 and age ≥ 50 years. The International Prognostic Score (IPS) was based on serum albumin ≤ 40 g/l, hemoglobin level ≤ 105 g/l, male sex, age ≥ 45, stage IV, leukocytosis (≥ $15 \times 10^9$ cells/l) and lymphocytopenia (≤ $0.6 \times 10^9$ cells/l). Chemoresistance was defined as radiological progression during the first-line ABVD chemotherapy. A complete response was defined as a no longer visible tumor after first-line ABVD treatment. The ethics committee of the Northern Ostrobothnia Hospital District approved the study design (reference number 42/2010).

## Immunohistochemical methods

Lymphoma samples collected from the patients at the time of diagnosis were fixed in formalin and embedded in paraffin. A representative tumor area from the paraffin blocks was cut in 3.5-µm sections and placed on SuperFrostPlus glass slides (Menzel-Gläser, Braunschweig, Germany). The slides were deparaffinized in xylene, rehydrated through graded alcohols and rinsed in distilled water. Next, the slides were microwaved for 15 minutes in citrate buffer solution, pH 6, to retrieve the epitopes and after 20 minutes' cooling at room temperature, the endogenous peroxidase activity was neutralized in peroxidase blocking solution (S2023, Dako, Glostrup, Denmark) for 5 minutes. The next step was blocking with normal horse serum (SIRT1, SIRT4 and Rif1) and normal goat serum (SIRT6 and MGMT), followed by incubation with primary antibodies (Table 2) in a humidity chamber at 4 °C overnight. Immunostaining was continued using Vectastain Elite ABC kits for goat or for rabbit antibodies (Vector Laboratories, CA, USA), according to the instructions of the manufacturer. Diaminobenzidine was used to detect the immunoreaction. Between all

stages of the immunostaining procedure, the slides were washed with PBS-Tween. Finally, the slides were counterstained with Mayer´s hematoxylin, dehydrated and mounted.

## Grading of immunohistochemical staining

Evaluation of immunostaining was performed by an experienced hematopathologist (KMH) together with another investigator (HB) blinded to the clinical data. Immunostaining was graded (i) separately in Reed–Sternberg (RS) cells and in the surrounding reactive cellular infiltrate; (ii) separately in nuclei and cytoplasm; and (iii) separately according to the extent (0–100%) and intensity of immunostaining (1, weak intensity; 2, moderate intensity; 3, strong intensity; 4, very strong immunostaining intensity).

## Statistical analysis

For statistical analyses, immunostaining intensity (0–4) was multiplied by the extent of immunostaining (0–100%), resulting in a continuous variable of 0–400. This continuous variable was used in all statistical analyses, using the Mann–Whitney test with the exception of survival analyses. Associations between protein expression and patient survival were analyzed by using the Kaplan–Meier method, and the statistical significance of differences was evaluated by using the log-rank test. In survival analyses, continuous expression variables were divided into two classes (low or high expression) based on the median expression of each variable. Disease-specific survival (DSS) was calculated from the date of diagnosis to the date of lymphoma-

specific death or to the last follow-up date. Relapse-free survival (RFS) was calculated from the date of diagnosis to the date of the first confirmed relapse of HL. Cox regression analysis was applied in multivariate analysis. Statistical analyses were carried out by using IBM SPSS Statistics 22.0.0.0 software (SPSS, Chicago, IL, USA) and the results were considered significant if the two-sided p-value was <0.05.

**Results**

The extent of SIRT1, SIRT4, SIRT6, MGMT and Rif1 expression in RS cells and in reactive cellular infiltrate are presented in Table 3 and examples of the immunostaining patterns are shown in Figure 1 and 2. Curiously, SIRT1 and SIRT6 expression were not observed in the nuclei of neoplastic RS cells even though reactive cellular infiltrate showed nuclear staining (Figure 2A, Figure 2C). Nuclear expression of RI1 was stronger in neoplastic RS cells (Figure 2E), while MGMT expression was stronger in nuclei of the reactive elements compared with RS cells.

High-level cytoplasmic SIRT6 staining in RS cells was associated with low (0–2) IPSs (p=0.001), and predicted lower probability of achieving a complete response to first-line ABVD chemotherapy (p=0.007). When evaluating the reactive cellular infiltrate, strong nuclear immunostaining of SIRT1 and SIRT6 was associated with both B-symptoms (p=0.012, p=0.026 respectively) and advanced stage (p=0.011, p=0.038 respectively). Strong cytoplasmic SIRT6 staining in reactive cellular infiltrate also predicted a poor benefit from first-line ABVD chemotherapy (p=0.021).

The expression of Rif1 in RS cells was associated with the achievement of a complete response after ABVD treatment (p=0.039). Low-level cytoplasmic MGMT staining in RS cells was associated with the presence of B-symptoms (p=0.031) and advanced stage (p=0.023).

There were highly significant correlations between expression levels of the studied proteins – mainly positive correlations between SIRT6 and Rif1 expression, which are presented in more detail in Table 4.

Survival analysis

High-level cytoplasmic SIRT6 expression in reactive cellular infiltrate was also associated with prolonged RFS in the whole patient population (p=0.040) (Figure 3A). When the patients were divided according to stage (limited or advanced) and the administration of radiotherapy, SIRT6 was a prognostic factor only in those with advanced-stage disease who had received radiotherapy (p=0.031) (Figure 3B). However, these associations were not confirmed in multivariate analysis. In the subgroup of advanced-stage patients who had received radiotherapy, none of the seven patients with high-level SIRT6 expression experienced relapse, compared with 7/14 (50%) patients with low-level expression. In other words, SIRT6 had a positive prognostic value of 50% and a negative prognostic value of 100% (p=0.047).

High-level nuclear Rif1 expression in RS cells was associated with prolonged RFS, but only in cases with advanced-stage disease (univariate analysis, p=0.032) (Figure 3C). In multivariate analysis, high-level nuclear Rif1 expression in cases with advanced-stage disease was a more significant predictor of favorable RFS (HR 8.596; 95% CI 1.604–46.073; p=0.012) than a high IPS (scores 0–2 versus scores 3–7, HR 5.207; 95% CI 1.108–19.351; p=0.036). When the patients were further divided according to therapy, nuclear Rif1 expression in RS cells had prognostic value only among the advanced-stage-disease patients who had received radiotherapy (p=0.0043) (Figure 3D). This subgroup was unfortunately too small to be analyzed in multivariate analysis. In this subgroup, 7/11 (63.6%) of the patients with low-level Rif1 expression suffered from relapse, compared with 0/9 (0%) patients with high-level Rif1 expression. Thus, Rif1 had a positive prognostic value of 63.6% and a negative prognostic value of 100% as regards developing relapse in this subgroup (p=0.0047).

Based on the results of survival analysis in connection with nuclear Rif1 expression in RS cells and cytoplasmic SIRT6 in reactive cellular infiltrate, these two variables were further combined to a single factor: 0 = Rif1 and SIRT6 expression below the median; 1 = Rif1 and/or SIRT6 expression above the median. Low-level expression of both Rif1 and SIRT6 predicted worse RFS in univariate analysis (p=0.021) (Figure 3E). However, in subgroups the significance remained only among those patients who had received radiotherapy (p=0.0073). Similarly, low-level expression of both Rif1 and SIRT6 predicted poor outcome in those with advanced-stage disease (p=0.002). Among the patients with both advanced-stage disease and radiotherapy received, the

significance was even more pronounced (p=0.000038) (Figure 3F). In multivariate analysis this combined variable was still significant as regards the radiotherapy-treated patients (HR 8.521; 95% CI 1.714–42.358; p=0.0088) when the stage (HR 9.395; 95% CI 9.395–46.935) was included in the analysis.

In line with the above, low-level Rif1/SIRT6 expression was associated with worse DSS in univariate analysis, but only in the patients treated with radiotherapy and with advanced-stage disease (p=0.034 for the whole population; p=0.024 for those with advanced-stage HL; p=0.011 for the patients treated with radiotherapy; p=0.015 for the patients with advanced-stage HL and radiotherapy). This observation could not be confirmed in multivariate analysis as a result of the low number of HL-related deaths.

**Discussion**

To the best of our knowledge, this is the first study in which the expression levels of SIRT1, 4 and 6, Rif1 and MGMT in HL patients have been assessed. Our results show that there are differences in the expression levels and location of SIRT1, 4 and 6 between neoplastic Hodgkin's RS cells and reactive cells, and Rif1 and MGMT also display some differences. The results also suggest that Rif1 and SIRT6 may have clinically significant prognostic and even predictive roles in cases of untreated HL. None of the patients with advanced-stage disease suffered relapse after radiation therapy if they had either high-level nuclear Rif1 expression in RS cells or high-level cytoplasmic SIRT6 expression in reactive cellular infiltrate.

According to the current data, high-level cytoplasmic SIRT6 expression is associated with chemoresistance and advanced-stage disease, which are two of the strongest indicators of an aggressive course of HL. Our results also suggested that high-level cytoplasmic SIRT6 expression in reactive cellular infiltrate was associated with prolonged survival in the whole cohort and especially in patients with advanced-stage disease who received radiotherapy. Although the sample size limited multivariate analysis, it is interesting to note that none of the advanced-stage patients with high-level SIRT6 expression suffered relapse during the follow-up period. The minimum follow-up time was 1 year 10 months.

Our results are in line with those of a recent study which suggested that overexpression of adenovirus-mediated SIRT6 in lung-cancer cells not only inhibited proliferation and enhanced apoptosis, but also led to radiosensitization [39]. Increased levels of the transcription factors HIF-1α (hypoxia-inducible factor 1, alpha subunit) and Myc have been connected with radioresistance [40,41]. SIRT6 inhibits both HIF-1α and Myc expression, which might be a reason for enhanced radiosensitivity in SIRT6-high tumors [14]. The mainly cytoplasmic location of SIRT6, however, contradicts this suggesting other mechanisms to be involved. Under oxidative stress, SIRT6 significantly stimulates DSB-repair mechanisms, mainly by stimulating PARP-1 [20]. PARP-1 inhibitors have been recently taken into clinical use in ovarian carcinoma *BRCA*-carriers [42]. There is also evidence that in nasopharyngeal carcinoma, PARP-1 inhibition improves radiosensitivity [43,44]. Additionally, SIRT6 knockdown mice display hypersensitivity to ionizing radiation due to potential defects in base excision repair [45]. HR and DSB repair are due to SIRT6`s promotion of

several enzymatic functions including PARP1 [45]. Misplacement of SIRT6 mainly into the cytoplasm could then lead to improved radiosensitivity due to attenuated DNA repair.

On the other hand, in breast cancer, increased SIRT6 expression has been linked to chemoresistance to paclitaxel and epirubicin [29]. The main mechanism of action of anthracyclines, such as doxorubicin, is to damage DNA by intercalating into DNA and also by creating reactive oxygen species [46,47]. We earlier demonstrated that high-level oxidative stress both in RS cells and reactive cellular infiltrate is associated with an aggressive course of HL [48]. In this study SIRT6 appeared to be more oncogenic than tumor-suppressing in cases of HL, especially among advanced-stage patients with radiotherapy administration, although we cannot prove causality on the basis of the results of this purely immunohistochemical study.

In addition to the above, low-level nuclear Rif1 expression in RS cells was associated strikingly with dismal RFS in patients with advanced-stage disease, especially in those in this category who received radiotherapy. In multivariate analysis Rif1 had more prognostic significance than the widely clinically adopted IPS system. Rif1 is potent in facilitating NHEJ. On the other hand, it also greatly suppresses HR in a BRCA1-mediated manner, causes a shift from HR to NHEJ and also sensitizes cell lines to ionizing radiation [6, 49, 50]. Of these two DSB repair mechanisms, HR has a critical role in the development of radioresistance [51, 52]. There are potential radiosensitizer compounds that inhibit the HR repair mechanism [53]. On the basis of our results, it may be hypothesized that Rif1 suppresses HR in RS cells treated with radiotherapy, which may lead to enhanced radiotherapy efficacy and ultimately to an

excellent patient outcome in those with advanced disease.

Rif1 and SIRT6 have not been previously assessed in the same study in humans. Both of them improve radiosensitivity, Rif1 mainly by reducing HR and making NHEJ more effective and SIRT6 by inhibiting HIF-1α and Myc transcription factors. In the current material the expression levels of SIRT6 and Rif1 correlated closely with each other. When nuclear Rif1 in RS cells and cytoplasmic SIRT6 in reactive cellular infiltrate were combined, high-level expression of either of the markers was associated with prolonged disease-specific survival, even in advanced-stage patients who received radiotherapy. Despite the limited material, the negative prognostic value of 100% was a very surprising result.

We conclude that Rif1 and SIRT6 expression levels should now be validated in a larger cohort of patients to confirm their prognostic roles. The role of radiotherapy is under debate as regards HL as a result of its long-term side effects and excellent outcome of the patients. The prognostic significance of Rif1/SIRT6 expression was observed only in the patients with advanced disease and who had received radiotherapy and the novel predictive factors may be especially important for this patient group.

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**Potential conflict of interest:**

The authors have no conflict of interests.

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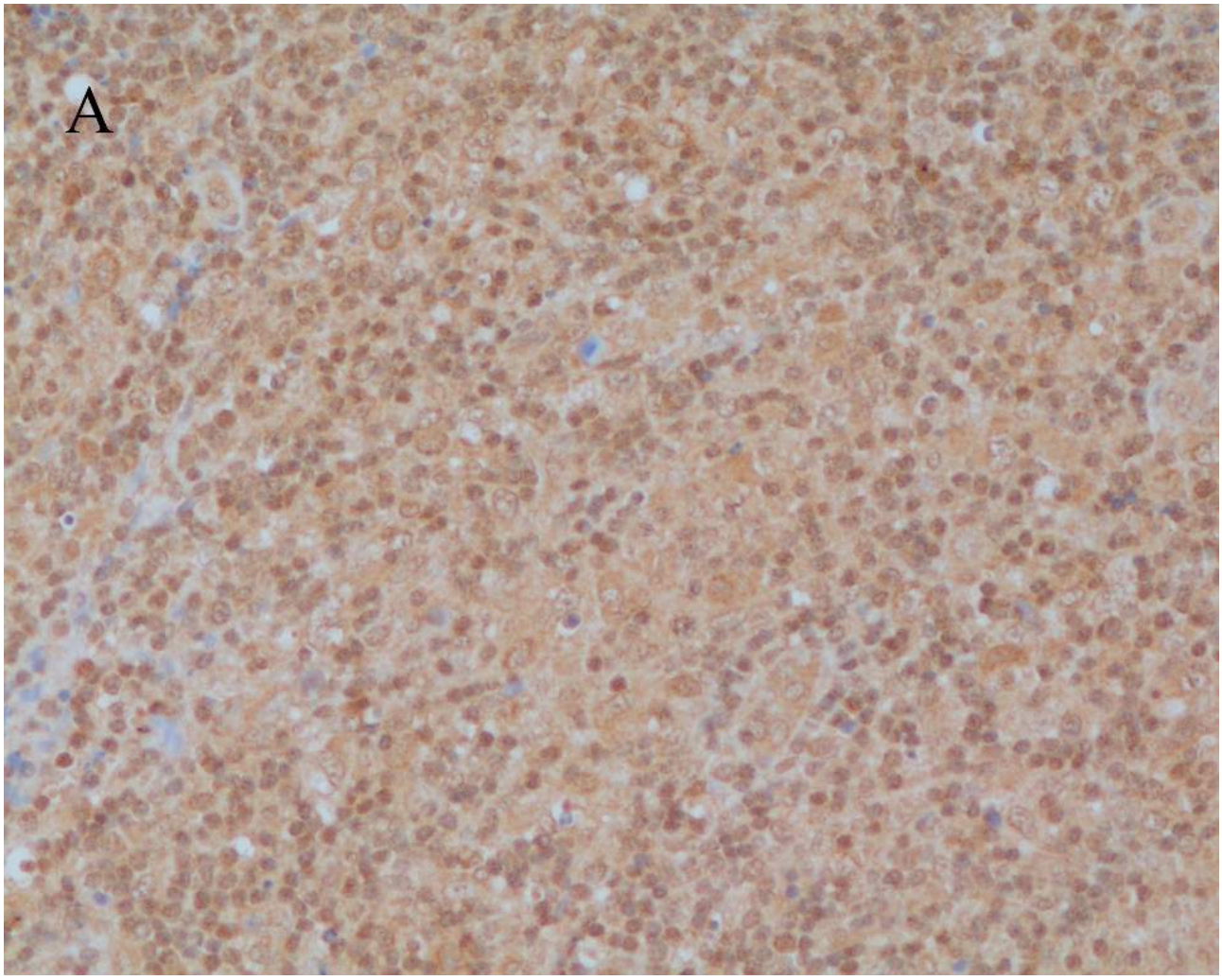
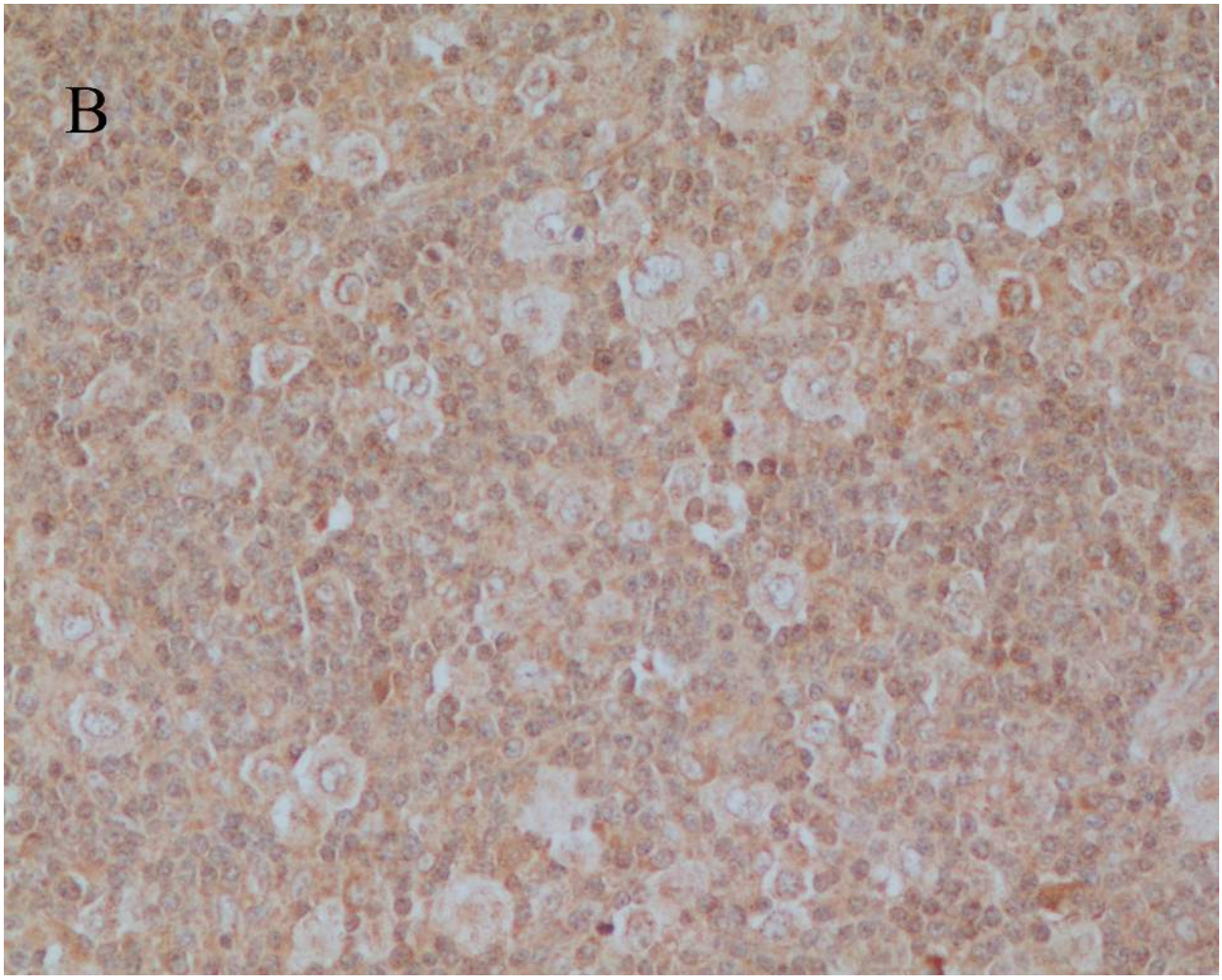
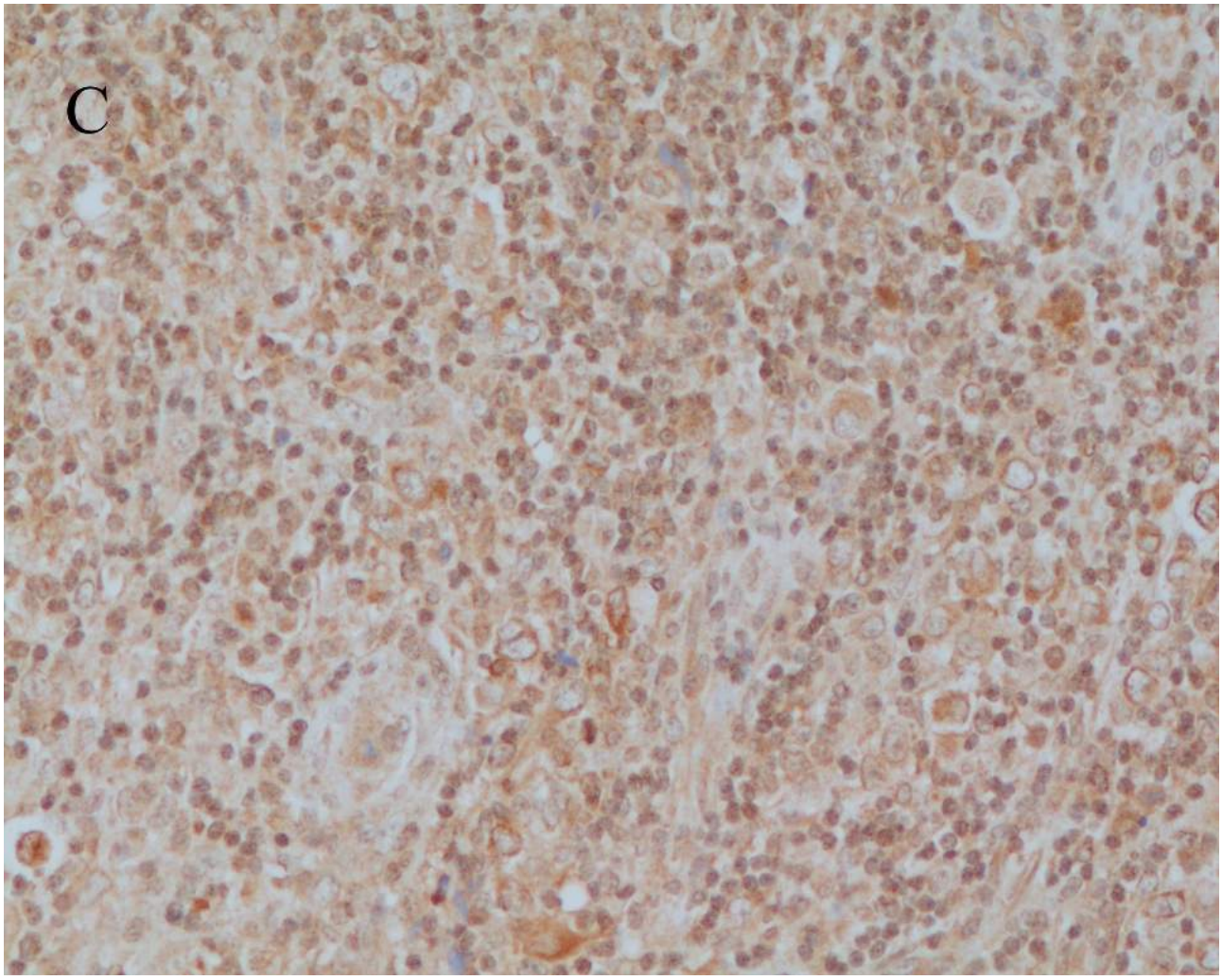
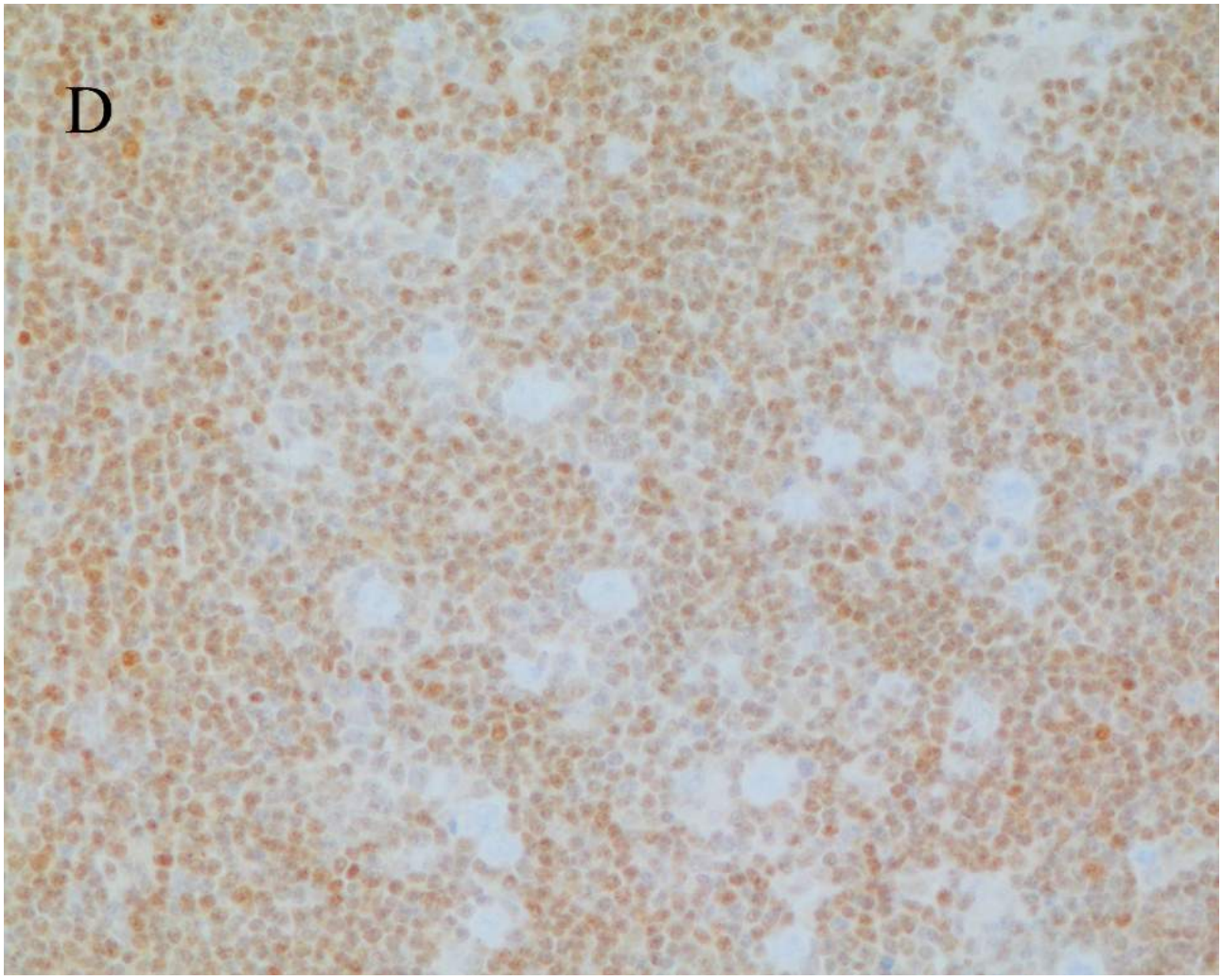
Figure 1. Immunostaining of SIRT1, SIRT6, MGMT and Rif1 expression in classical Hodgkin's lymphoma. All figures are at ×20 magnification.

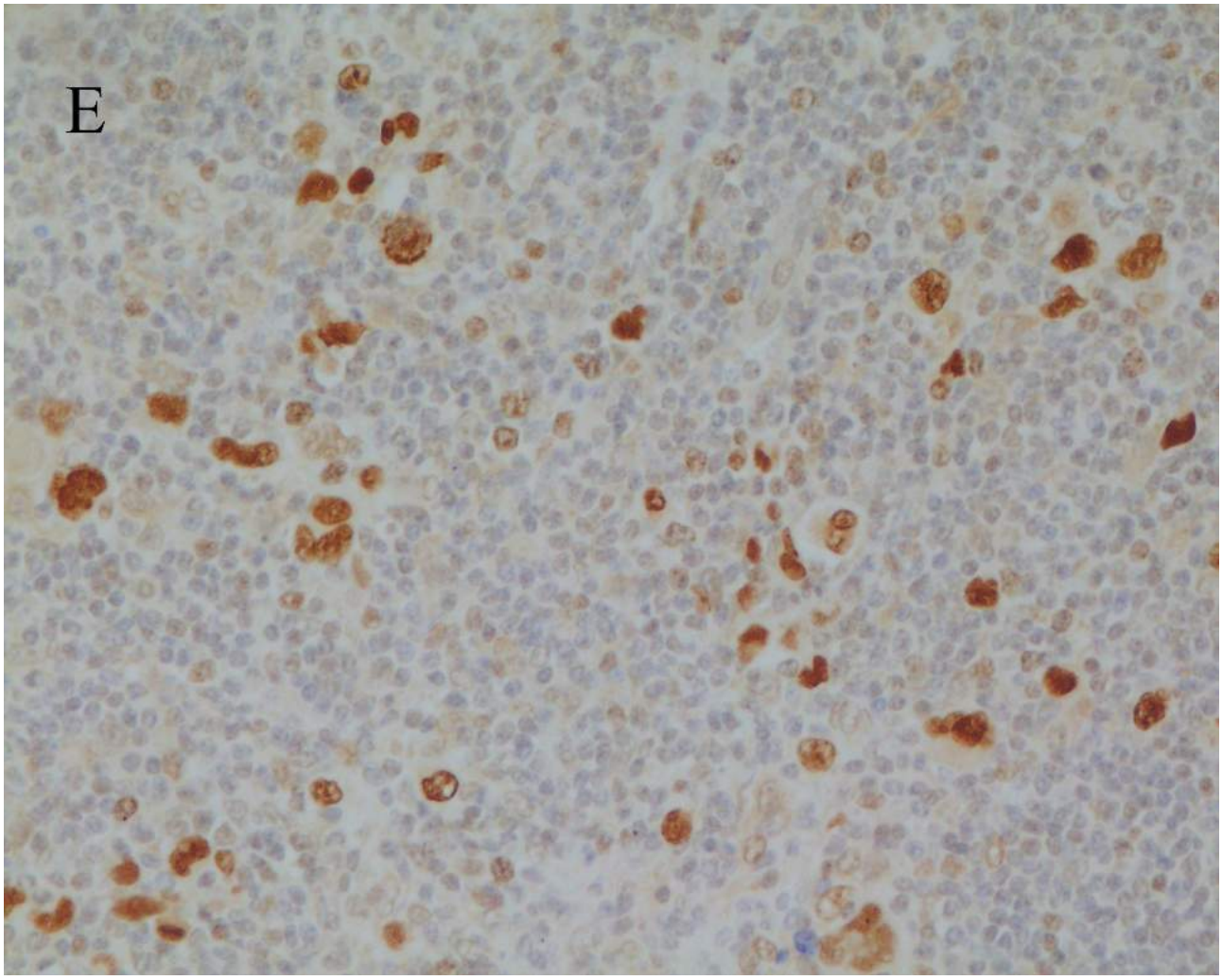
A. Cytoplasmic SIRT1 expression was weak in Reed–Sternberg (RS) cells. In the cellular infiltrate, SIRT1 showed strong nuclear staining.
B. Very weak SIRT6 expression in the cytoplasm of RS cells. Expression in the cytoplasm of reactive cellular infiltrate was strong.
C. Very weak SIRT6 expression in the cytoplasm of RS cells. Strong nuclear immunostaining in most of the reactive cellular infiltrate cells.
D. In RS cells, MGMT immunostaining was negative. Strong nuclear immunostaining in most of the reactive cellular infiltrate cells.
E. Mostly very strong nuclear Rif1 expression in RS cells. Staining was partly granular. In the reactive cellular infiltrate there was some nuclear expression.
F. Mainly very strong nuclear and weak to moderate cytoplasmic Rif1 expression in the RS cells. There was also some nuclear expression in the reactive cellular infiltrate.


A


B


C


D


E

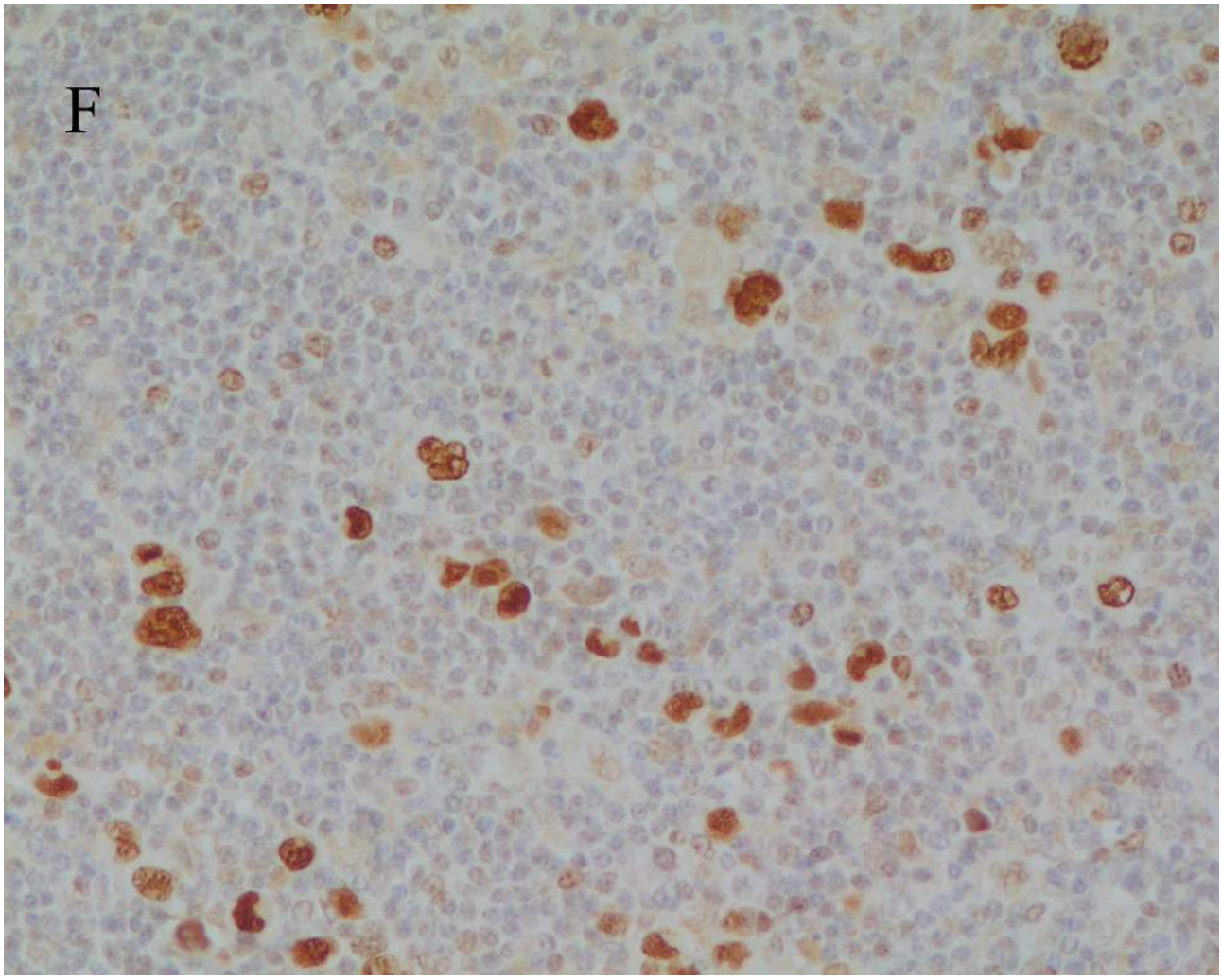
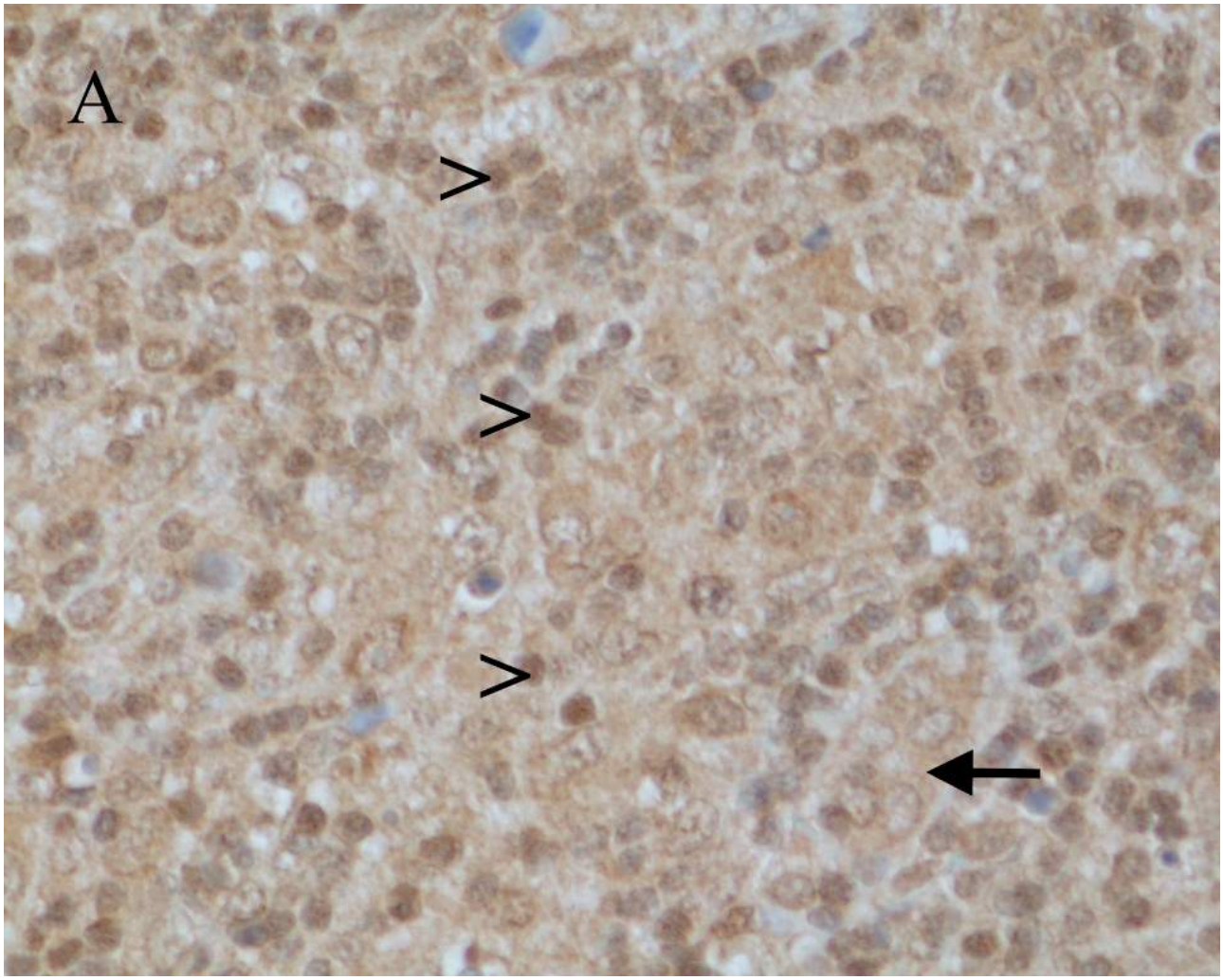
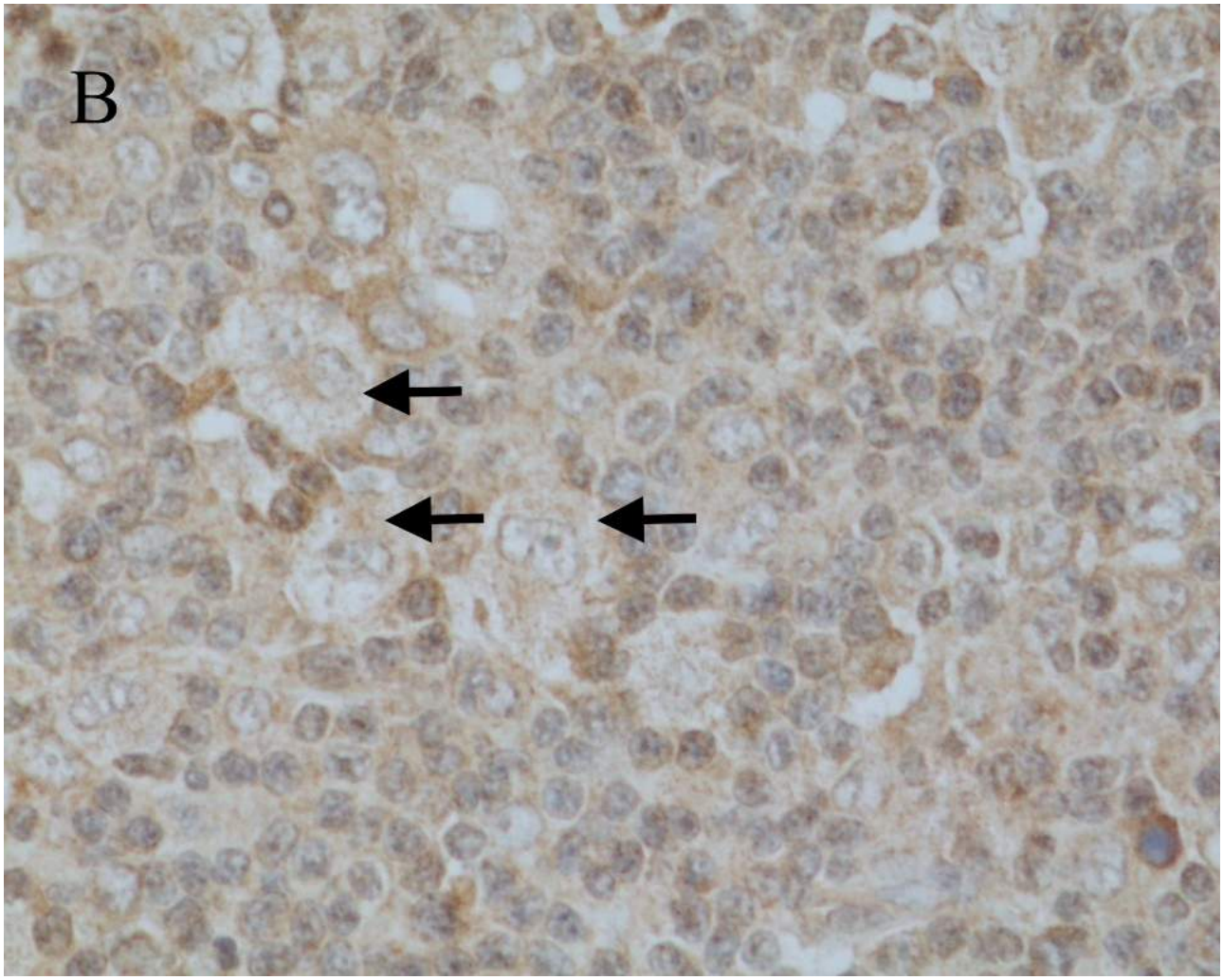
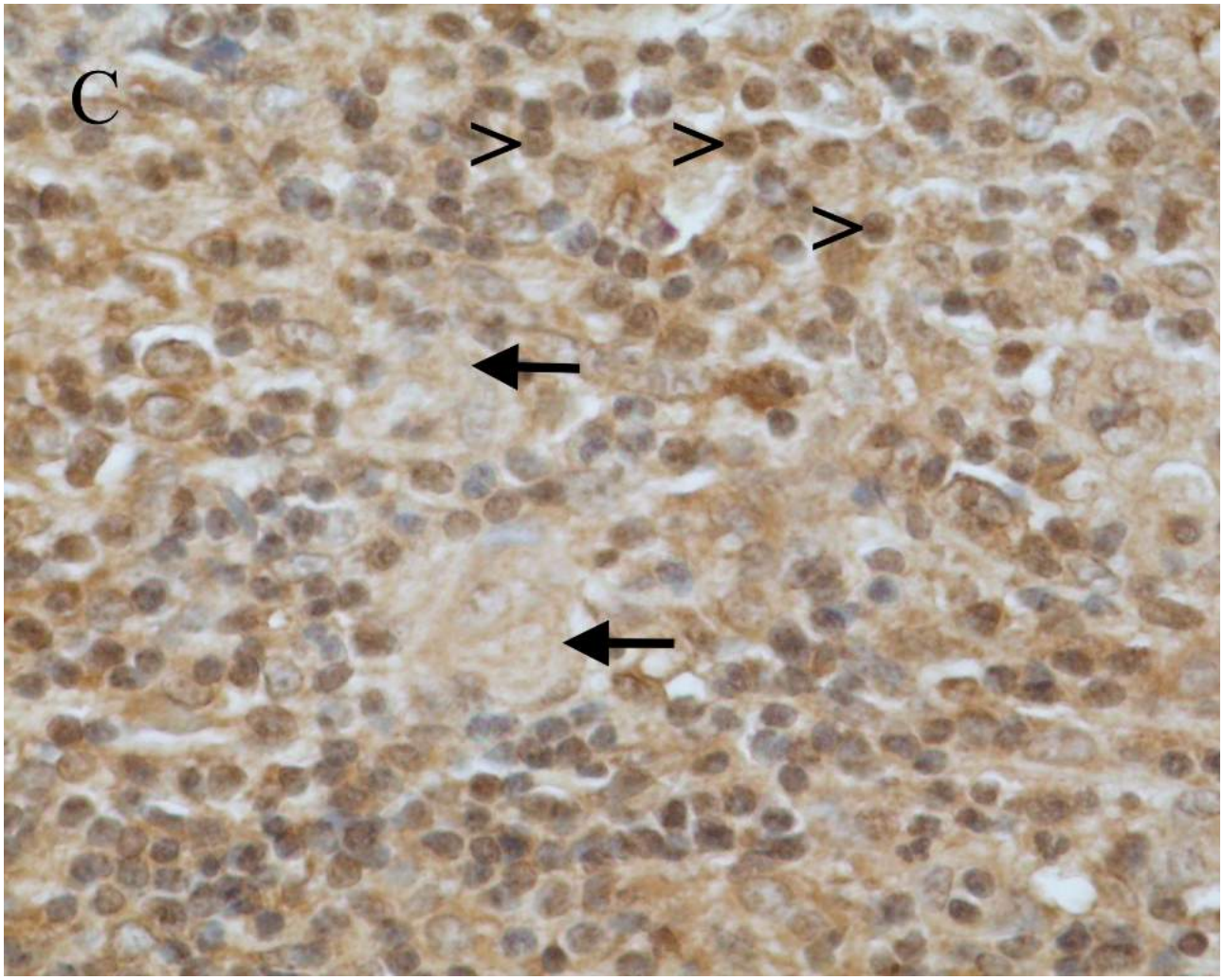

F

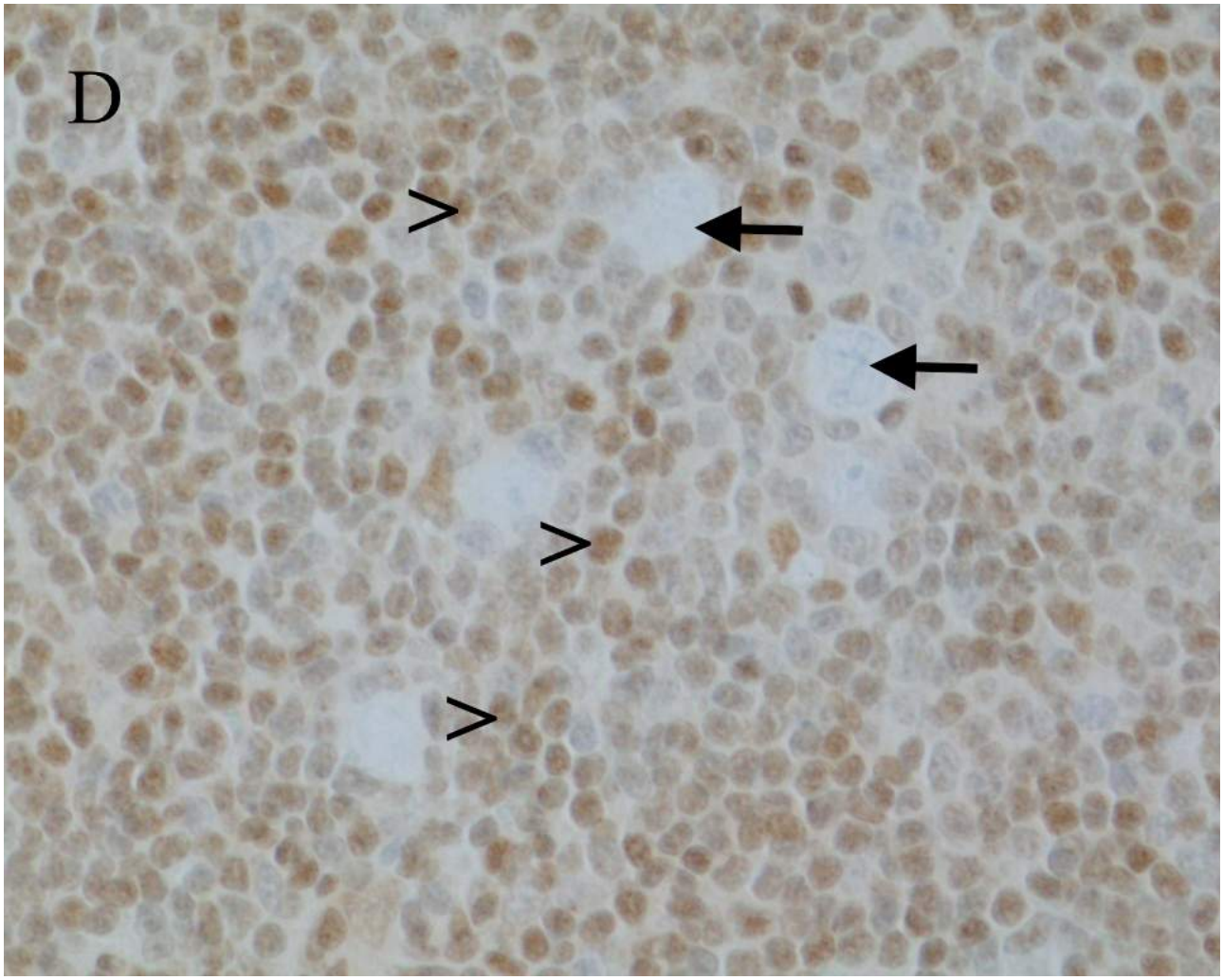
Figure 2. Immunostaining of SIRT1, SIRT6, MGMT and Rif1 expression in classical Hodgkin's lymphoma with arrows and arrowheads to indicate subcellular localization patterns. All figures are at ×40 magnification.

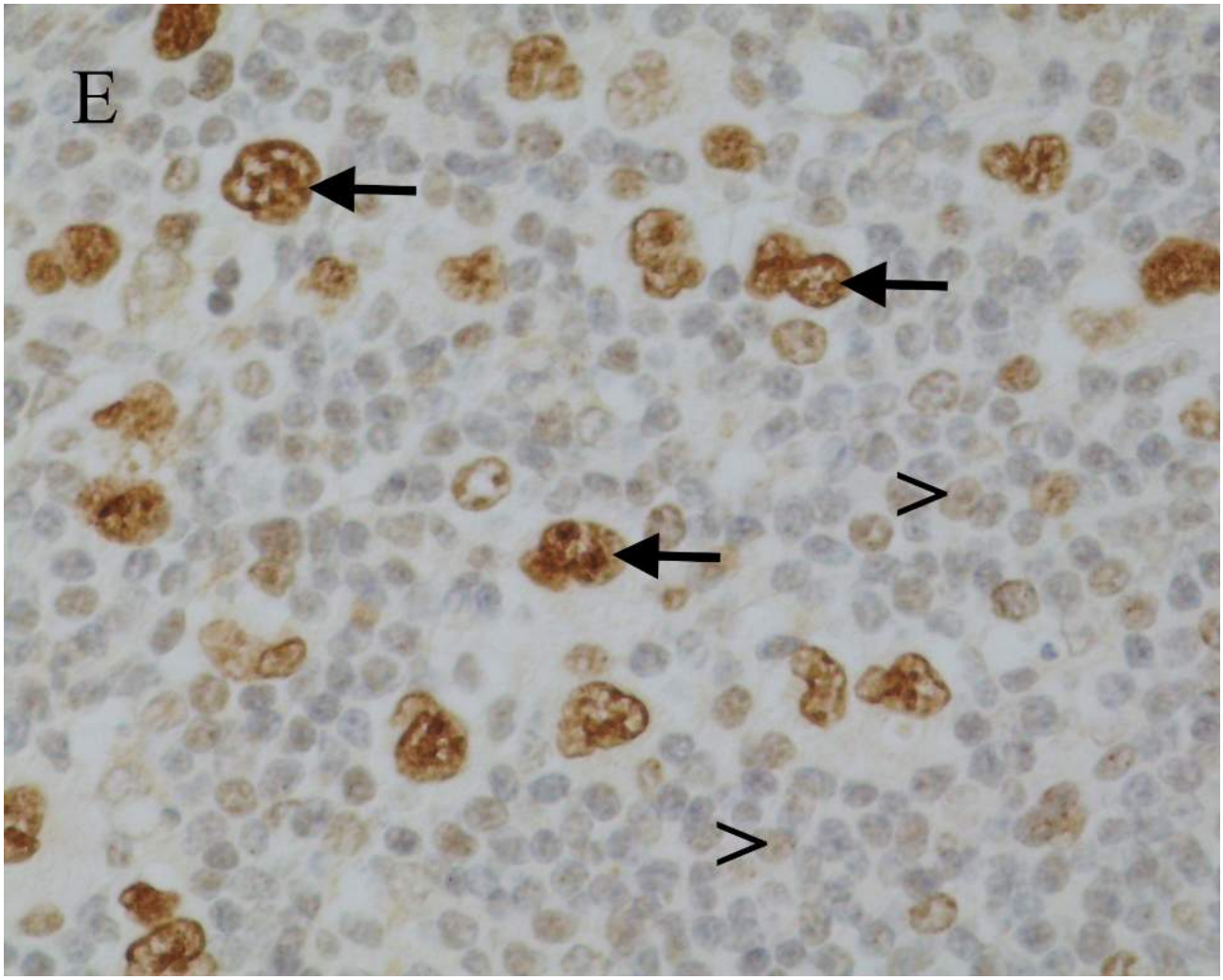
A. Arrow indicates subcellular localization of low cytoplasmic SIRT1 expression in RS cells. Arrowheads indicates strong nuclear staining in reactive cellular infiltrate.
B. Arrows show weak SIRT6 cytoplasmic expression in RS cells.
C. Arrows show weak SIRT6 cytoplasmic expression in RS cells and arrowheads indicates strong nuclear SIRT6 expression in reactive cellular infiltrate.
D. Arrows demonstrate negative MGMT expression of RS cells. Arrowheads indicates moderate nuclear expression in cellular infiltrate.
E. Arrows point strong nuclear Rif1 expression in RS cells. Arrowheads point weak cytoplasmic Rif1 expression in reactive cellular infiltrate.
F. Arrows indicate strong nuclear Rif1 expression in RS cells. Arrowheads point moderate nuclear expression in reactive cellular infiltrate.


A


B


C


D


E

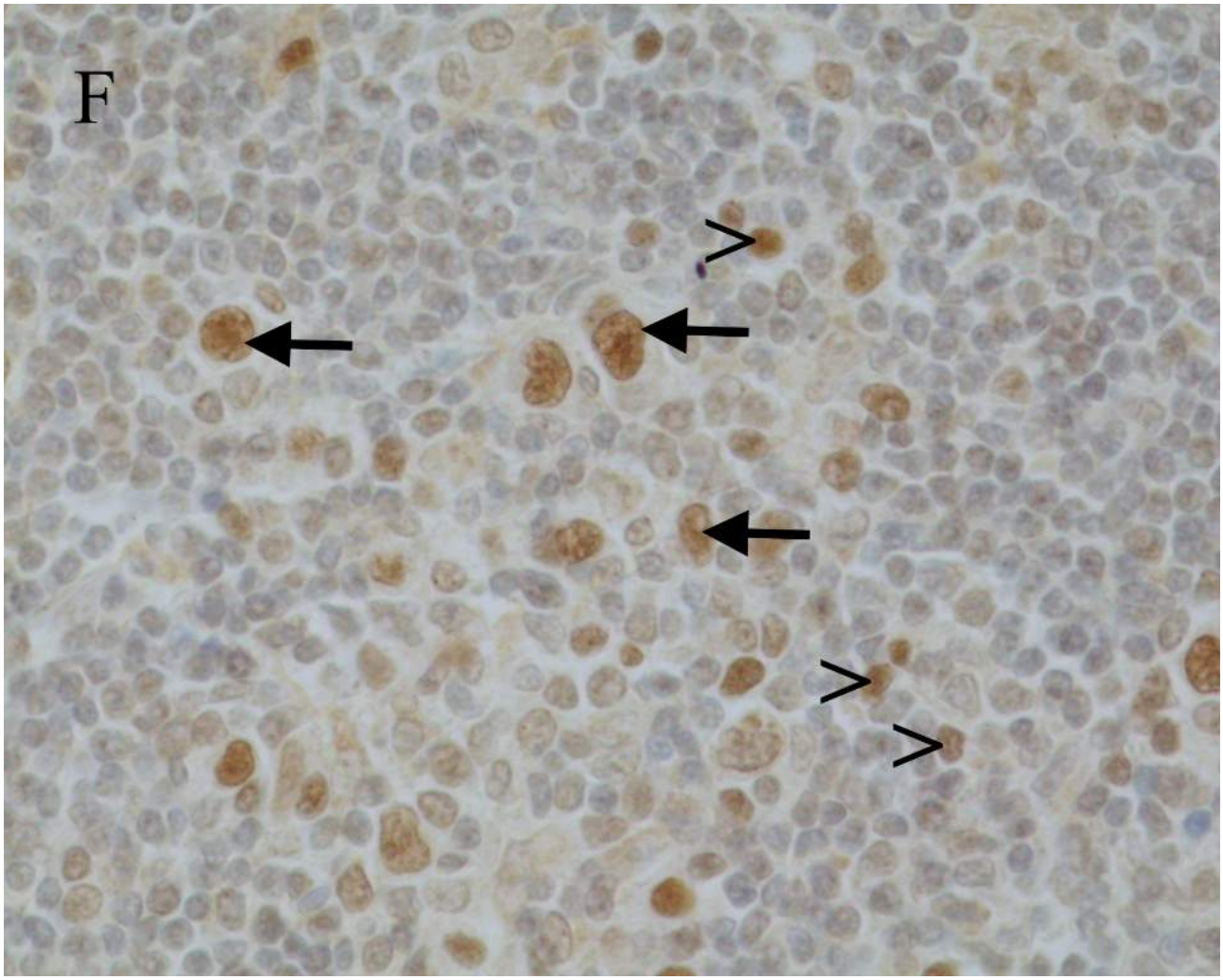
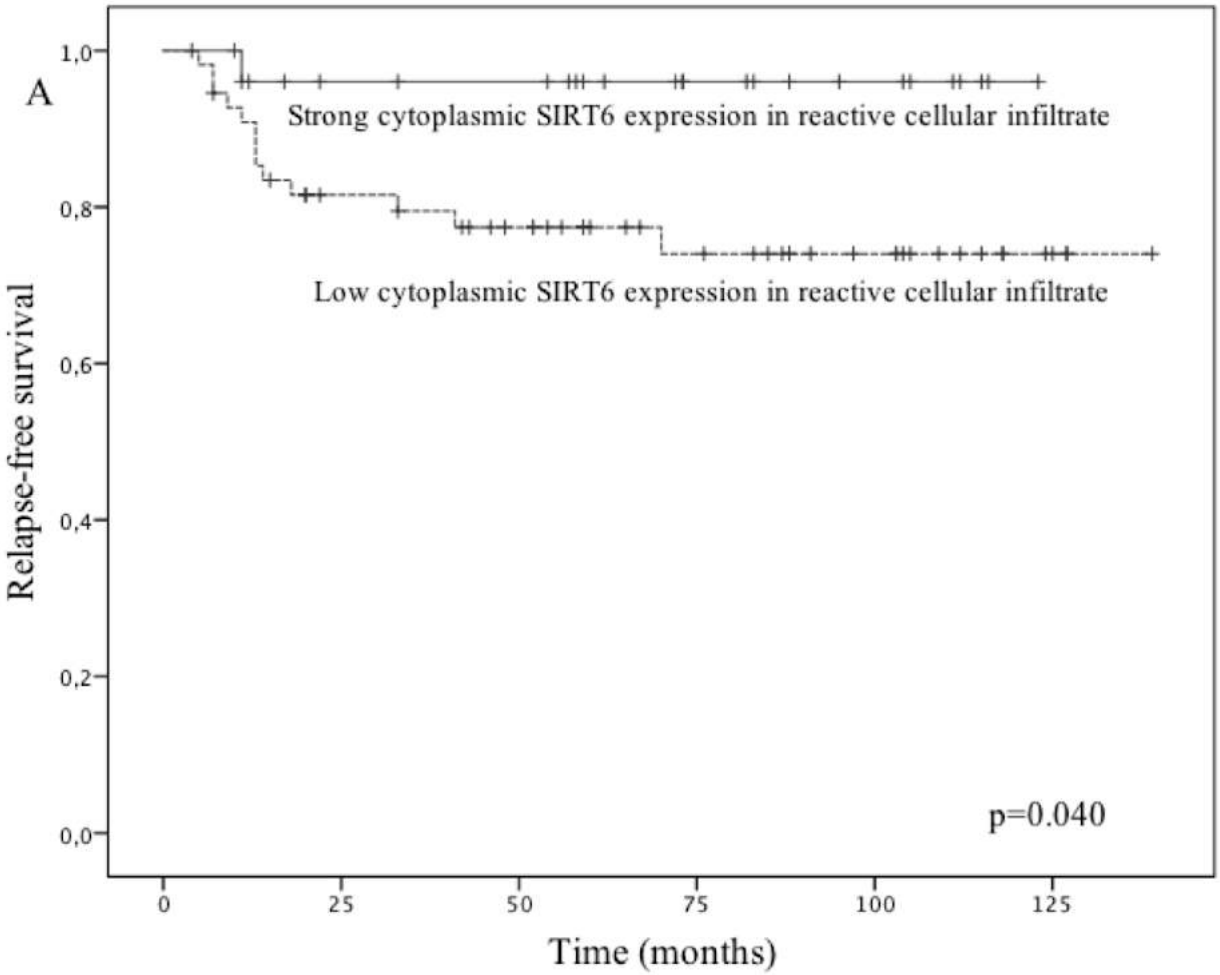
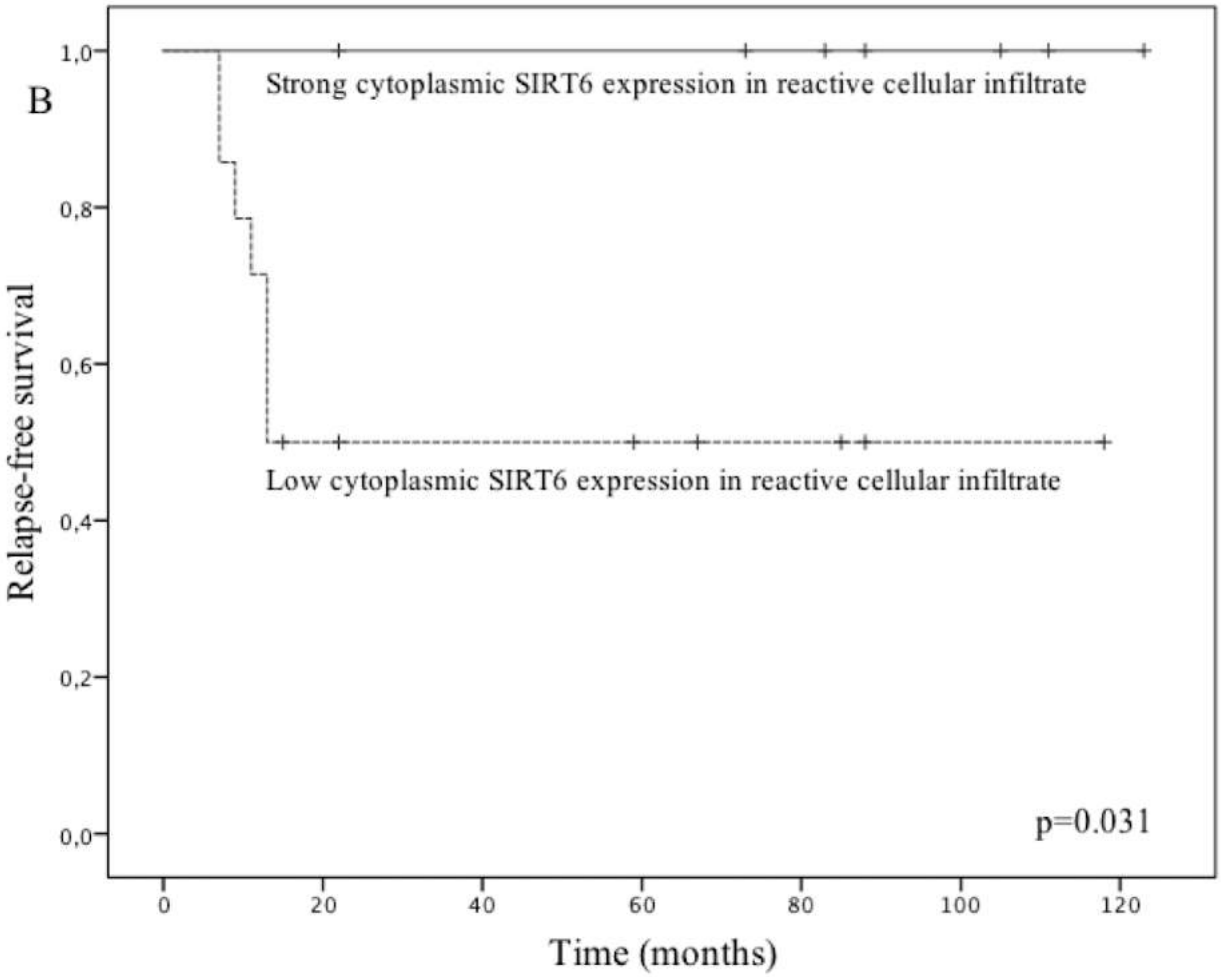
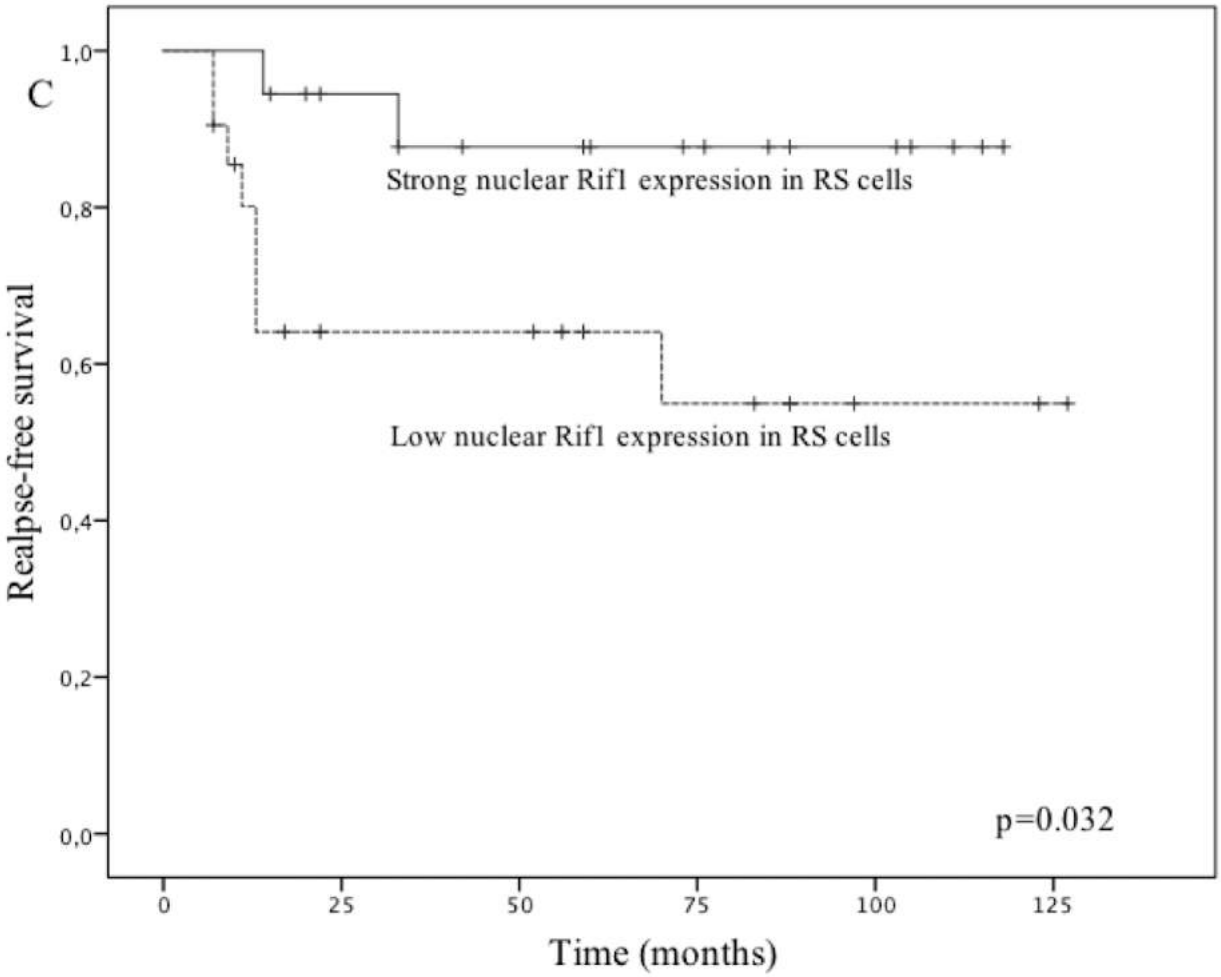

F

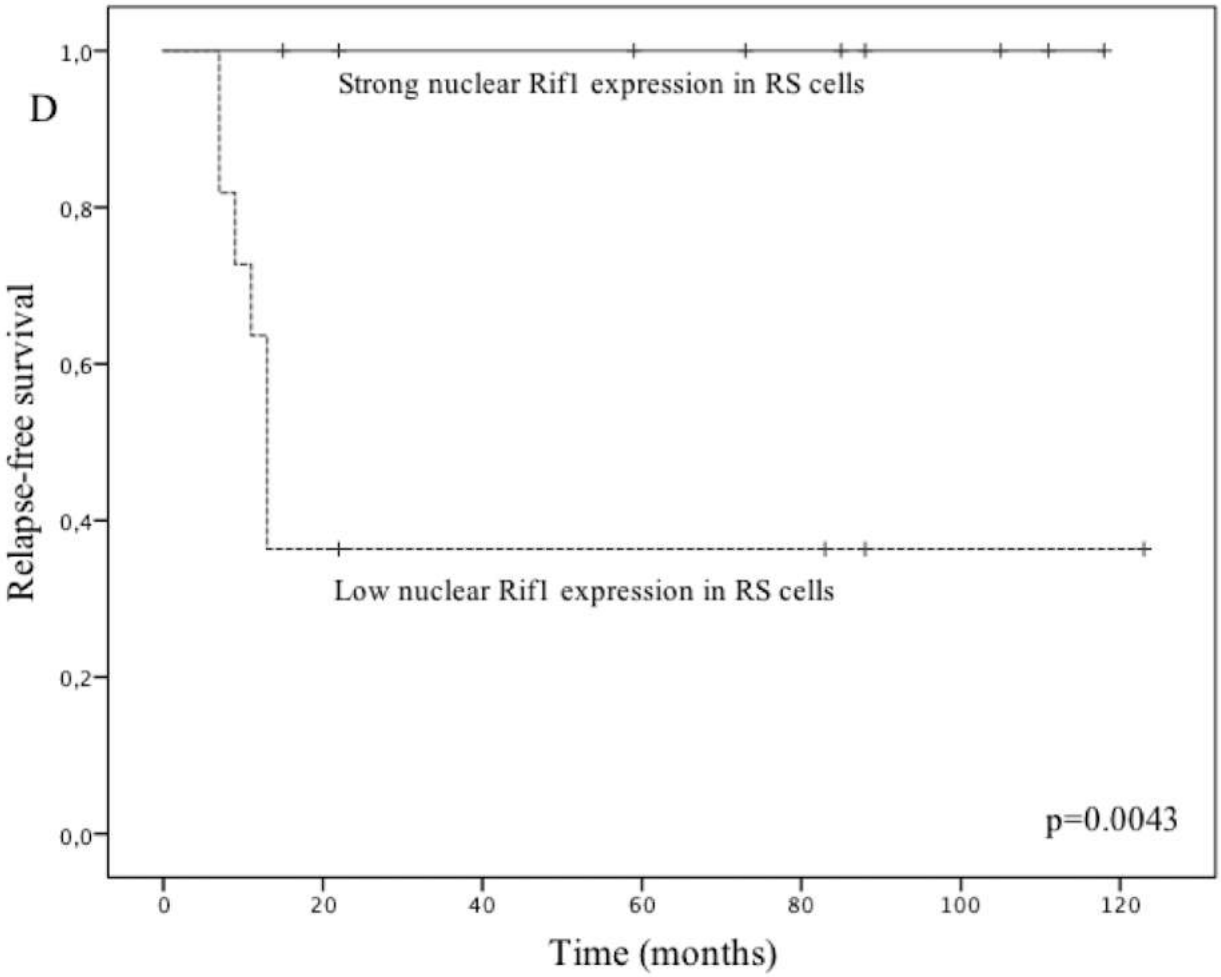
Figure 3. Kaplan–Meier analysis showing relapse-free survival. Crosses indicate censored cases.

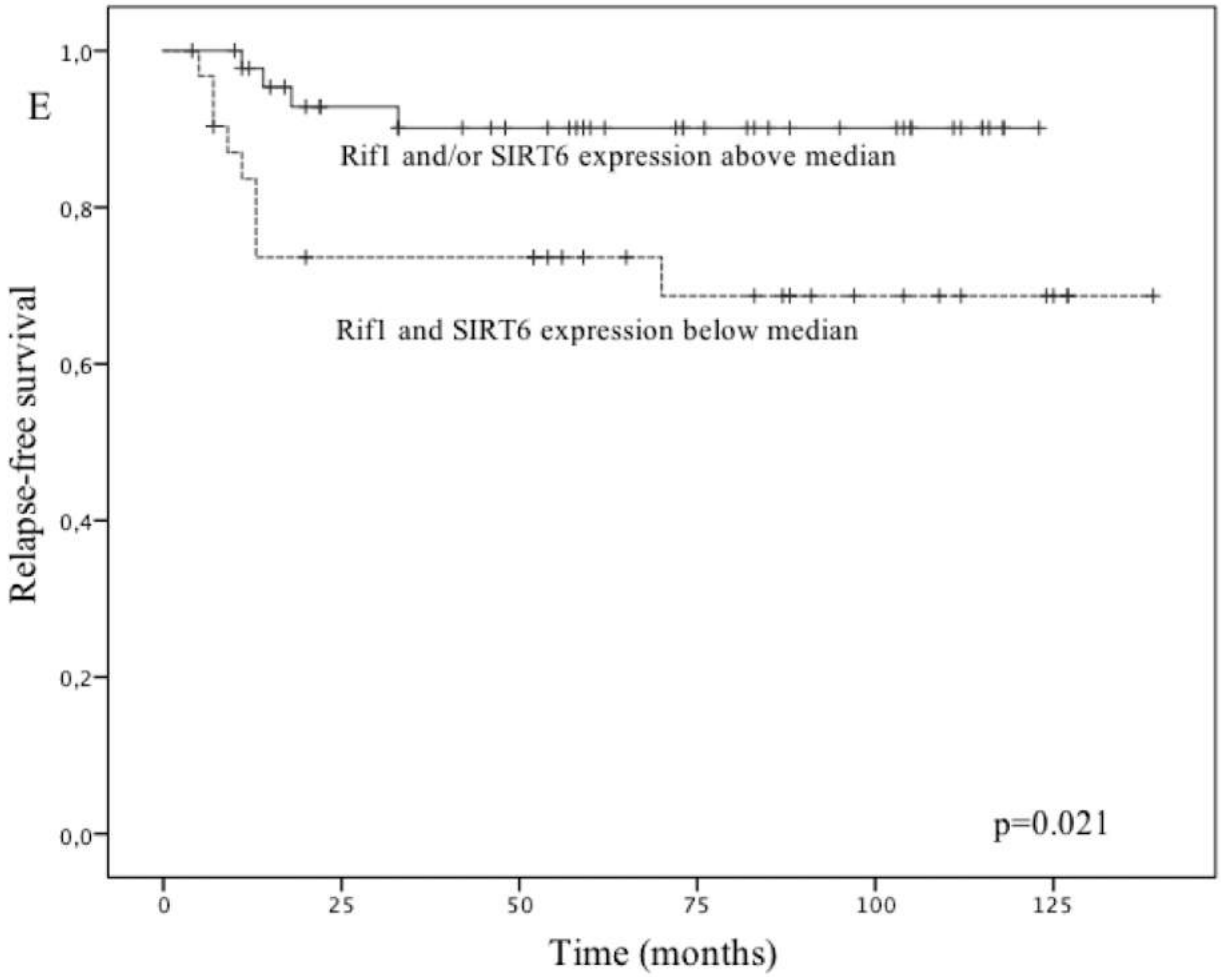
A. Cytoplasmic SIRT6 expression in reactive cellular infiltrate in the whole cohort
B. Cytoplasmic SIRT6 expression in reactive cellular infiltrate with advanced-stage classical Hodgkin Lymphoma (cHL) who were treated with radiotherapy
C. Nuclear Rif1 expression in Reed–Sternberg cell with advanced-stage cHL
D. Nuclear Rif1 expression in Reed–Sternberg cell with advanced-stage cHL who were treated with radiotherapy
E. New variable (nuclear Rif1 expression in RS cells and cytoplasmic SIRT6 in reactive cellular infiltrate) in the whole cohort
F. New variable (nuclear Rif1 expression in RS cells and cytoplasmic SIRT6 in reactive cellular infiltrate) separately in the patients with advanced-stage cHL who were treated with radiotherapy.


A
Strong cytoplasmic SIRT6 expression in reactive cellular infiltrate
Low cytoplasmic SIRT6 expression in reactive cellular infiltrate
p=0.040
Relapse-free survival
1,0
0,8
0,6
0,4
0,2
0,0
0
25
50
75
100
125
Time (months)


B
Strong cytoplasmic SIRT6 expression in reactive cellular infiltrate
Low cytoplasmic SIRT6 expression in reactive cellular infiltrate
p=0.031
Relapse-free survival
1,0
0,8
0,6
0,4
0,2
0,0
0
20
40
60
80
100
120
Time (months)


C
1,0
0,8
0,6
0,4
0,2
0,0
Realpse-free survival
Strong nuclear Rif1 expression in RS cells
Low nuclear Rif1 expression in RS cells
p=0.032
0
25
50
75
100
125
Time (months)


D
Strong nuclear Rif1 expression in RS cells
Low nuclear Rif1 expression in RS cells
p=0.0043
Relapse-free survival
1,0
0,8
0,6
0,4
0,2
0,0
0
20
40
60
80
100
120
Time (months)


E
Relapse-free survival
1,0
0,8
0,6
0,4
0,2
0,0
Rif1 and/or SIRT6 expression above median
Rif1 and SIRT6 expression below median
p=0.021
0
25
50
75
100
125
Time (months)

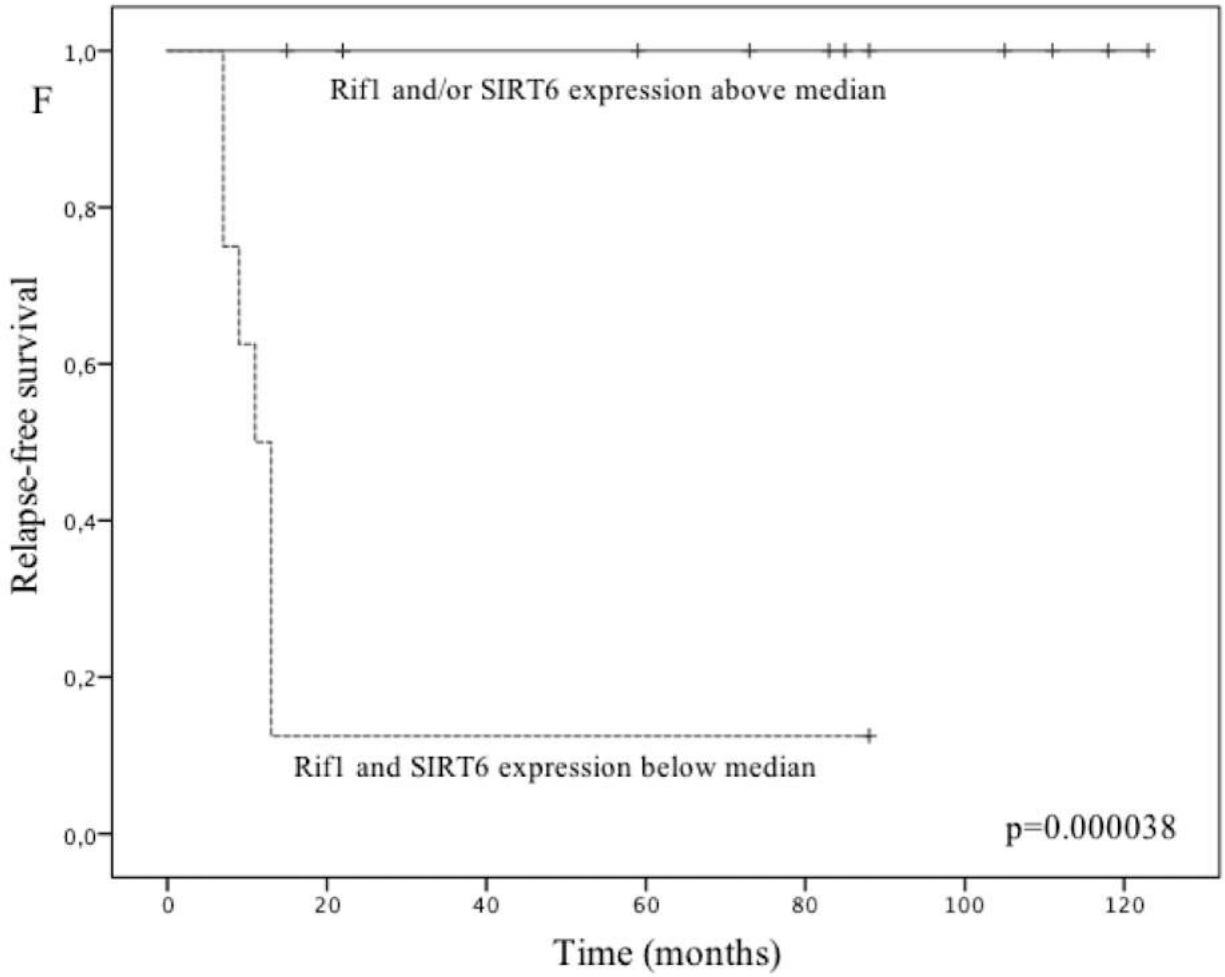

F
Relapse-free survival
1,0
0,8
0,6
0,4
0,2
0,0
Rif1 and/or SIRT6 expression above median
Rif1 and SIRT6 expression below median
p=0.000038
0
20
40
60
80
100
120
Time (months)

Table 1. Demographics of the patients.

| | Limited stage | | Advanced stage | | Total | |
|---|---|---|---|---|---|---|
| | n | % | n | % | n | % |
| Median age at diagnosis, years (range) | 32 | (16-85) | 24 | (17-70) | 27 | 16-85 |
| Sex | | | | | | |
| Male | 21 | 52.3% | 22 | 53.7% | 43 | 50.1% |
| Female | 23 | 47.7% | 19 | 46.3% | 42 | 49.9% |
| Histology (ICD-10 code) | | | | | | |
| C81.1 Nodular sclerosis classical Hodgkin lymphoma | 35 | 79.5% | 28 | 68.3% | 63 | 74.1% |
| C81.2 Mixed cellularity classical Hodgkin lymphoma | 4 | 9.1% | 10 | 24.4% | 14 | 16.5% |
| C81.7 Other (classical) Hodgkin lymphoma | 4 | 9.1% | 2 | 4.9% | 6 | 7.1% |
| C81.9 Hodgkin lymphoma, unspecified | 1 | 2.3% | 1 | 2.4% | 2 | 2.4% |
| B-symptoms | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Absent | 42 | 95.5% | 11 | 26.8% | 53 | 62.4% |
| Present | 2 | 4.2% | 30 | 73.2% | 32 | 37.6% |
| Stage | | | | | | |
| Limited | | | | | 44 | 51.8% |
| Advanced | | | | | 41 | 48.2% |
| Limited stage risk factors | | | | | | |
| None | 18 | 40.9% | | | | |
| ≥ 1 | 26 | 59.1% | | | | |
| International Prognostic Score | | | | | | |
| 0-2 | | | 31 | 75.6% | | |
| 3-5 | | | 10 | 24.4% | | |
| WHO performance status ≥ 1 | 5 | 11.4% | 23 | 56.1% | 28 | 32.9% |

| | | | |
|---|---|---|---|
| Number of ABVD cycles received | | | |
| 2-3 | 6 13.6% | 0 0 % | 6 7.1% |
| 4-6 | 26 59.1% | 0 0 % | 26 30.6% |
| 6-7 | 12 27.3% | 25 61.0% | 37 43.5% |
| 8 | 0 0 % | 16 39.0% | 16 18.8% |
| Complete response with first-line ABVD | | | |
| None | 12 29.5% | 15 36.6% | 29 31.9% |
| Yes | 31 70.5% | 26 63.4% | 61 67.8% |
| Radiotherapy | | | |
| No | 4 9.1% | 22 53.7% | 26 30.6% |
| Yes | 40 90.9% | 19 46.3% | 59 69.4% |
| Complete response after radiothrapy | | | |
| No | 3 7.5% | 3 15.8% | 6 10.2.% |

| | | | | | | |
|---|---|---|---|---|---|---|
| Yes | 37 | 92.5% | 16 | 84.2% | 53 | 89.8% |
| Relapse | | | | | | |
| No | 40 | 90.0% | 30 | 73.2% | 76 | 89.4% |
| Yes | 4 | 10.0% | 11 | 26.8% | 15 | 17.6% |
| Deaths | | | | | | |
| Lymphoma-specific deaths | 1 | 2.3% | 6 | 14.6% | 7 | 8.2% |
| Deaths due to other causes | 3 | 6.8% | 1 | 2.4% | 4 | 4.7% |

Table 2. Immunohistochemical methods.

| Primary antibody | Source of primary antibody | Dilution | Immunostaining method |
|---|---|---|---|
| Anti-Rif1 (ab134812) | Abcam, Cambridge, UK | 1 / 200 | Vectastain ABC kit PK6100, Vector Laboratories Inc., CA, USA |
| Anti-Sirt1 (ab166821) | Abcam, Cambridge, UK | 1 / 200 | Vectastain ABC kit PK6100, Vector Laboratories Inc., CA, USA |
| Anti-Sirt4 (ab10140) | Abcam, Cambridge, UK | 1 / 250 | Vectastain ABC kit PK6100, Vector Laboratories Inc., CA, USA |
| Anti-Sirt6 (PA5-13225) | Thermo Fisher Scientific, Rockford, IL, USA | 1 / 100 | Vectastain ABC kit PK6100, Vector Laboratories Inc., CA, USA |
| Anti-MGMT (ab108630) | Abcam, Cambridge, UK | 1 / 750 | Vectastain ABC kit PK6100, Vector Laboratories Inc., CA, USA |

Table 3. Percentages of cases showing expression of SIRT1, SIRT4, SIRT6, Rif1 and MGMT.

| | Reed–Sternberg cells | | Reactive cellular infiltrate | |
|---|---|---|---|---|
| | Nuclei | Cytoplasm | Nuclei | Cytoplasm |
| SIRT1 | 0% | 100% | 35% | 89% |
| SIRT4 | 0% | 95.6% | 13% | 95.6% |
| SIRT6 | 0% | 100% | 56% | 96.7% |
| Rif1 | 96.5% | 82.1% | 81.7% | 74.4% |
| MGMT | 74.7% | 13.2% | 98.9% | 0% |

Table 4. Two-sided p-values and Pearson correlation coefficients between sirtuin and Rif1 expression. MGMT is not included in the Table due to the lack of any correlation. NS=no statistical significance; RSC=Reed–Sternberg cell; RCI=Reactive cellular infiltrate.

| Rif1 | | RSC-Nuclear | RSC-Cytoplasmic | RCI-Nuclear | RCI-Cytoplasmic |
|---|---|---|---|---|---|
| SIRT1 | RSC-Cytoplasmic | p=0.044, +0.227 | NS | NS | NS |
| | RCI-Nuclear | NS | NS | p=0.02, +0.264 | NS |
| | RCI- Cytoplasmic | NS | NS | NS | p=0.048, +0.226 |
| SIRT4 | RSC-Cytoplasmic | NS | NS | NS | NS |
| | RCI-Nuclear | NS | NS | NS | NS |
| | RCI- Cytoplasmic | NS | p=0.036, -0.235 | NS | NS |
| SIRT6 | RSC-Cytoplasmic | p=0.009, +0.292 | NS | NS | NS |
| | RCI-Nuclear | NS | p<0.001, -0.397 | p<0.001, +0.424 | NS |
| | RCI- Cytoplasmic | p=0.027, +0.248 | p=0.001, +0.357 | NS | NS |