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Altered Actin and Immunoglobulin Cμ Expression in Nitrogen Mustard-Resistant Human Burkitt Lymphoma Cells

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Raji-HN2 is a B cell lymphoma (Burkitt lymphoma) line that was made resistant to nitrogen mustard. The drug-resistant phenotype was accompanied by changes in gene expression. The expression of four unrelated genes was examined by Northern blot analysis. Raji-HN2 cells were found to contain about twice the number of actin mRNA found in Raji cells. Both cell lines were found to contain equivalent amounts of β2-microglobulin, c-myc oncogene, and immunoglobulin Cμ mRNAs. The Cμ mRNA was, however, larger in size in Raji-HN2 cells. Alterations in actin and Cμ mRNAs in Raji-HN2 cells were not due to gene amplification or rearrangement because Southern blot analysis revealed no changes in the genomic organization of these genes. The increased actin mRNA content was correlated with an increased actin content of Raji-HN2 cells. The F-actin (stained with 7-nitrobenz-2-oxa-1,3-diazolylphallacidin) content of single cells was quantitated in a meridian interactive laser cytometer. Raji-HN2 cells contained about twice the amount of F-actin present in the parental Raji cells. Similar results were obtained when large populations, 10^6 cells each, were examined in a flow cytometer.

Key words: drug resistance, c-myc oncogene, β2-microglobulin, meridian laser cytometer

Tumor cells often acquire resistance to drugs that are used in cancer chemotherapy [1]. Numerous tissue culture cell lines have been developed to study the mechanism(s) of drug resistance in vitro. One such cell line, Raji-HN2, a Burkitt lymphoma cell line made resistant to nitrogen mustard (HN2), was developed to study the mechanism of resistance to alkylating agents [2]. HN2 is a bifunctional alkylating agent that interacts with DNA to form interstrand crosslinks which, if not removed, lead to cell death [3]. We reported that HN2 induced fewer cross-links in the DNA of Raji-HN2 than that of the parental Raji cells [4]. The reduced HN2-induced DNA cross-linking may be due to efficient repair of the DNA damage in Raji-HN2 cells. These cells have altered

Abbreviations used: HN2, nitrogen mustard [2-chloro-N-(2-chloroethyl)-N-methylethanamide]; NBD-phallacidin, 7-nitrobenz-2-oxa-1,3-diazolylphallacidin; 1×SSPE, 0.18 M NaCl, 10 mM NaPO4, pH 7.7, 1 mM EDTA.

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chromatin and increased topoisomerase II activity, both of which could play a role in DNA repair [4,5]. Because an altered chromatin would likely affect gene expression, we investigated the expression of three classes of unrelated genes to further our understanding of the Raji-HN2 cell system. The first class included the housekeeping genes actin [6,7] and β2-microglobulin [8,9]. The c-myc oncogene was investigated because this oncogene is believed to be the causative agent of Burkitt lymphoma [10,11]. The third class was represented by the immunoglobulin heavy μ chain gene that is expressed only by B lymphocytes [12].

MATERIALS AND METHODS

Cells

Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Raji-HN2 cells were treated weekly with 10 μM HN2 to maintain the resistant phenotype [2,4]. For comparative studies, both cell lines were harvested simultaneously in the exponential growth phase.

Preparation of Cells for Quantitation of Actin

Cells were seeded (2×10^5/ml) in two-chamber Lab-Tek slides. The medium was gently removed by aspiration and cells were fixed with 1% paraformaldehyde/glutaraldehyde in Dulbecco's PBS containing 15 mM HEPES. The fixative contained 100 μg/ml L-c-lysophosphatidylcholine palmitoyl (Sigma, St. Louis, MO) and 330 nM NBD-phallacidin (7-nitrobenz-2-oxa-1,3-diazolylphallacidin) (Molecular Probes, Inc., Junction City, OR) to permeabilize the cells and stain the actin filaments, respectively [13,14]. After 45 min at 22°C, the fixative/stain was replaced with fresh fixative/stain and the cells were incubated for an additional 45 min. The cells were then gently rinsed twice with Dulbecco's PBS, overlaid with a coverslip in a glycerol:water (9:1) mixture, and examined in a meridian interactive laser cytometer. The argon-ion laser of a meridian ACAS470 interactive laser cytometer was adjusted to visible output (488 nm) at a power setting of 100 mW. The beam was filtered by a neutral density filter and 485/20 nm bandpass filter before entering the microscope. A 510 laser barrier and 515 epifluorescent dichroic filter were used in the microscope and a 530/30 bandpass filter was placed in front of PMT1.

Cells were similarly prepared in vials, instead of slides, for study by flow cytometry. Measurements were performed in a Coulter EPICS 753 flow cytometer (Coulter Electronics, Hialeah, FL). The argon-ion laser was adjusted to 500 mW output power. The forward-angle light scatter detector was filtered by 2.0 neutral density filter. The right-angle light scatter signal was selected by a 488 nm longpass dichroic with a 488 nm bandpass filter in front of the photomultiplier. The laser blocking filters were 515 nm longpass interference and glass filters placed in sequence. A 525 nm bandpass filter was placed in front of the fluorescence photomultiplier tube. Cellular debris were gated out of the analysis based on the forward- (FALS) and right-angle (RALS) light scatter signals.

RNA Analysis

Cells were lysed with 4.5 M guanidine isothiocyanate and the lysate was underlaid with 10 ml of 5.7 M CsCl and centrifuged (Beckman SW28 rotor, 22000 rpm, 20°C, 20 h) [15]. The RNA pellet was dissolved in water and fractionated on an oligo-dT column

to yield poly(A) RNA [15]. Two micrograms of poly(A) RNA was denatured with 2.2 M formaldehyde (15 min/65°C) and electrophoresed in a 1.5% agarose gel containing 1 M formaldehyde as described [15]. The fractionated RNA was blotted directly onto a Zetaprobe nylon membrane (Biorad, CA) with 0.2 M NaOH for 1.5 h. The blot was neutralized with 2× SSPE (1× SSPE = 0.18 M NaCl, 10 mM NaPO4, pH7.7, 1 mM EDTA) for 10 min, washed with 0.5× SSPE, 0.1% SDS for 1 h at 65°C, and prehybridized for at least 3 h at 42°C in hybridization buffer lacking dextran sulfate (see below).

DNA Analysis

DNA was extracted from proteinase K-digested nuclei with phenol [15]. After digestion with restriction enzymes (2 U/μg DNA, 6 h at 37°C), the DNA was fractionated in a 1% agarose gel. The gel was shaken gently in 0.25 N HCl for 20 min, transferred to 0.4 M NaOH for 15 min, and blotted overnight to Zetaprobe membrane with 0.4 M NaOH [16]. The blot was then processed as described above.

Hybridization to Probes

The following human probes were used: a) β actin cDNA, 2 kb insert [6]; b) β2-microglobulin cDNA, 0.62 kb insert [9]; c) c-myc cDNA (Ryc 7.4) containing exons 2 and 3 [17]; and d) 1.2 kb EcoRI fragment of genomic Cμ [18]. ^{32}P-labeled probes were prepared by nick-translation according to manufacturer's instructions (New England Nuclear, Boston, MA). Labeled probes were denatured for 5 min in boiling water, quick-chilled, and added to hybridization solutions (5× SSPE, 33% formamide, 200 μg/ml heparin, 0.2% SDS, 0.2% sodium pyrophosphate, 6% dextran sulfate). Hybridization was at 42°C for 48–72 h. The blots were then washed under high-stringency conditions (final wash at 65°C with 0.1× SSPE for 30 min) [15] and exposed to X-ray films at −70°C.

Protein Analysis

Cells were lysed with SDS and analyzed in a 10–18% polyacrylamide gradient gel using the buffer system described by Laemmli and Favre [19]. After electrophoresis the gel was fixed with 50% MeOH/10% acetic acid, stained with Coomassie blue, and destained with several changes of 10% MeOH/7% acetic acid. Purified rabbit muscle actin (mol. wt. 43,000, Sigma, St. Louis, MO) was electrophoresed in parallel to serve as marker.

RESULTS

Actin

Examination by light microscopy showed that Raji-HN2 cells were generally larger and more heterogeneous in size than the parental Raji cells. Transmission electron microscopy revealed that the nuclei of Raji-HN2 cells were spherical whereas those of Raji cells had numerous indentations and were smaller (unpublished observations). An increase in nuclear size was also observed for a sarcoma cell line made resistant to adriamycin, a drug that interacts with chromatin [20]. Because of the difference in morphology between Raji and Raji-HN2 cells, we wanted to determine whether actin, a major component of the cytoskeleton, was altered in Raji-HN2 cells. Both cell lines grow

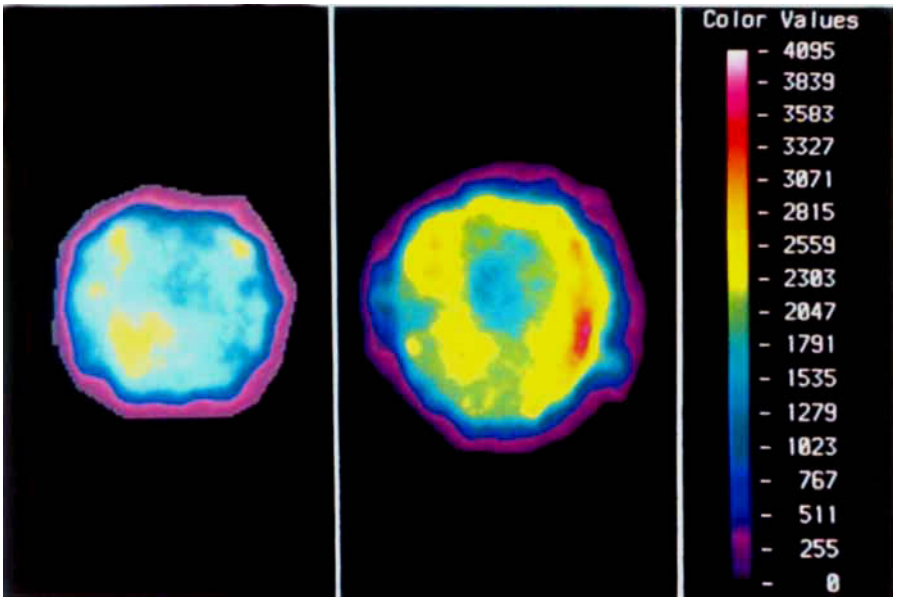

Fig. 1. Pseudocolor digitized computer images of cells stained with NBD-phallacidin. Raji-HN2 and Raji cells were fixed and stained with the actin filament specific stain NBD-phallacidin and analyzed in a meridian interactive laser cytometer. Each color was assigned a range of values (shown on right side) that were directly related to fluorescence intensity. Each image is representative of the images obtained from each group of 20 cells from two separate experiments.

as suspension cultures. We used a meridian interactive laser cytometer to quantitate the actin content of single cells labeled with NBD-phallacidin, an actin filament specific stain [13,14]. Pseudocolor images of a Raji-HN2 and a Raji cell stained with NBD phallacidin are presented in Figure 1. The distribution of fluorescent-labeled actin was similar for both Raji and Raji-HN2 cells, but the intensity of the fluorescence was significantly higher in Raji-HN2 cells. Arbitrary color values are shown on the right side of Figure 1, and the areas of greatest fluorescence intensity are areas where there is the highest degree of NBD-phallacidin binding to actin filaments. These images are representative of two separate experiments in which 20 cells were analyzed per experiment. The distribution of fluorescence/pixel intensity for 20 cells is shown in Figure 2. Most Raji cells had about one-half the fluorescence/pixel intensity of Raji-HN2 cells. A similar 2.2-fold difference in fluorescence/pixel intensity between Raji and Raji-HN2 cells was obtained when large samples, 1×10^6 cells each, were analyzed in a flow cytometer (Table I).

Actin exists as two major forms: nonpolymerized globular, or G-actin, and polymerized filaments, or F-actin [7]. The results presented above suggest that Raji-HN2 cells contained more F-actin than Raji cells. Next, we compared the total actin content of Raji and Raji-HN2 cells by gel analysis of cellular proteins. The gels were stained with Coomassie blue and scanned in a densitometer. Actin is easily identifiable as a major polypeptide with a molecular weight of 43,000 daltons. Because Raji-HN2 cells are larger and may contain more protein than Raji cells, the amount of actin was normalized against polypeptides which were present in equivalent amounts in the

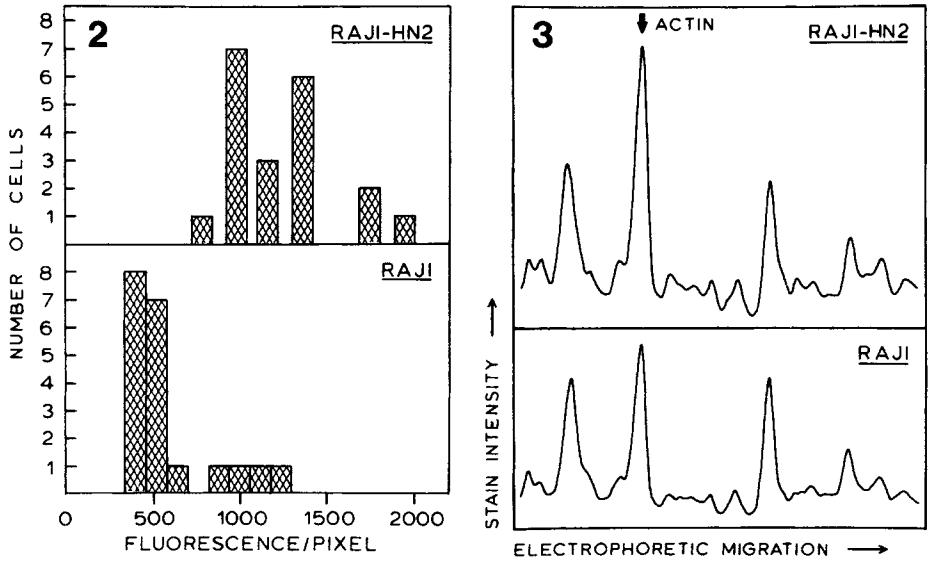

Fig. 2. Quantitation of actin filaments stained with NBD-phallacidin. Raji-HN2 and Raji cells were fixed, stained, and analyzed in a meridian interactive laser cytometer. 20 cells of each line were analyzed and the average fluorescence/pixel, in arbitrary units, was 1,131 ± 307(SD) for Raji-HN2 and 506 ± 275(SD) for Raji cells.

Fig. 3. Gel scan of Raji and Raji-HN2 actin. Cells were lysed with SDS, fractionated in a polyacrylamide gel, and polypeptides were stained with Coomassie blue. Purified rabbit skeletal actin was electrophoresed in parallel to serve as marker (arrow). The part of the gel containing actin was scanned in a densitometer. The area under the Raji-HN2 actin peak was 1.7-fold higher than that of Raji cells. One representative experiment of three shown.

TABLE I. Quantitative Fluorescence of F-Actin in Raji and Raji-HN2 Cells*

	Fluorescence intensity (arbitrary units)	
Experiment	Raji	Raji-HN2
1	48.6	99.1
2	41.1	112.3
3	54.7	80.0
Mean (±SD)	48.1 (±6.8)	97.1 (±16.2)

*Cells were fixed and stained as described in Materials and Methods and analyzed using a flow cytometer to quantify the amount of F-actin labeled with NBD-phallacidin. Three separate sets of cells, each containing 10^6 cells, were analyzed.

samples of both cell lines (Fig. 3). Raji-HN2 cells were found to contain about 1.7-fold more actin than Raji cells.

To quantitate actin mRNA, the autoradiograms of Northern blots (Fig. 4) were scanned and the areas under the actin mRNA peaks measured by planimetry. Raji-HN2 cells were found, in two separate experiments, to contain 2.2- and 2.3-fold more of actin mRNA (normalized against the same amount of RNA analyzed) than Raji cells.

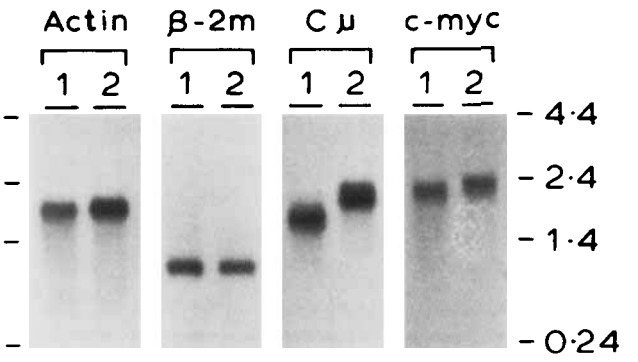

Fig. 4. Northern blot analysis. 2 μg of poly(A) RNA from Raji (1) and Raji-HN2 (2) cells were fractionated on an agarose gel, blotted to Zetaprobe membrane, and hybridized to nick-translated probes. After each analysis, the hybridized probe was stripped off by incubating the blot in boiling water for 20 min. The blot was then hybridized to another probe. The positions and sizes (in kb) of RNA molecular weight markers, electrophoresed in parallel, are shown on the right. The results were reproduced in another experiment. β-2m, β2-microglobulin.

Raji-HN2 cells were established after repeated treatments of Raji cells with a sublethal conc entration of HN2 [2]. Because DNA is a major target of HN2, the increased actin mRNA in Raji-HN2 cells could have reflected transcription from amplified or rearranged actin genes induced by HN2 [21,22]. To test this hypothesis, DNA was extracted from the cells and fractionated by electrophoresis after digestion with restriction enzymes. The fractionated DNA fragments were transferred to a nylon membrane which was hybridized with an actin probe. The results presented in Figure 5 show that similar amounts (determined by scanning autoradiograms) of the actin probe hybridized to identical fragments of Raji and Raji-HN2 DNA. Thus, the actin genes of Raji-HN2 cells do not appear to have been amplified or rearranged.

β2-Microglobulin

We compared the expression of another house-keeping gene, β2-microglobulin, in Raji and Raji-HN2 cells. β2-microglobulin is a small protein that is noncovalently associated with the major histocompatibility complex [8] that is present in all vertebrate cells. Both β2-microglobulin mRNA and genomic sequences were unaltered in Raji-HN2 cells (Figs. 4, 5).

c-myc

Raji cells were derived from a Burkitt lymphoma in which the c-myc oncogene was translocated from one chromosome 8 to the γ-constant region gene of the immunoglobulin heavy-chain locus [23,24]. Although both the normal and translocated c-myc oncogenes are expressed, most of the mRNA are derived from the translocated allele [24,25]. Northern blot analysis of the RNA from Raji and Raji-HN2 cells revealed that similar amounts of c-myc mRNA were present in both cell lines (Fig. 4). Restriction enzyme analysis suggested that the genomic c-myc sequences were not altered in Raji-HN2 cells (Fig. 5).

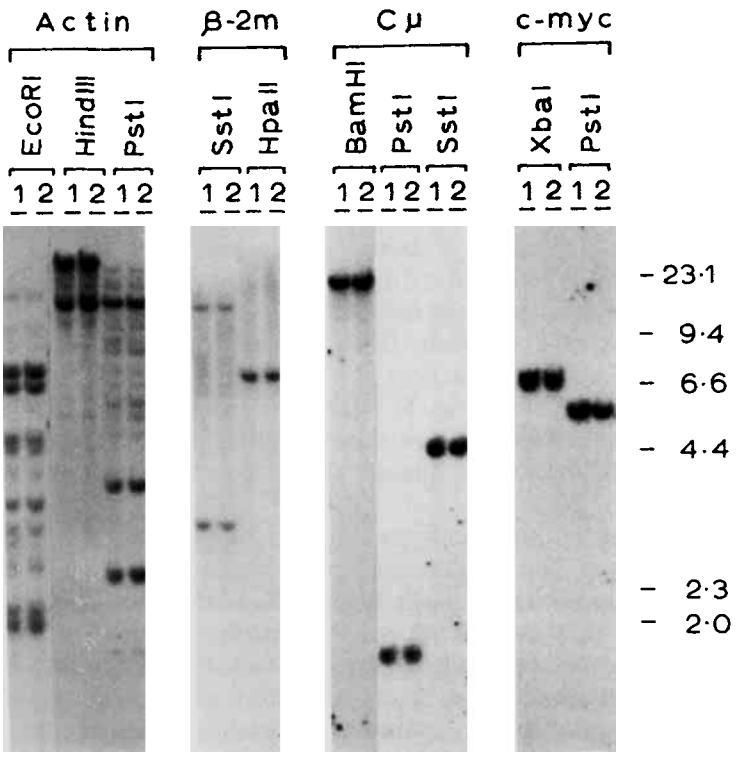

Fig. 5. Southern blot analysis of actin, c-myc, and Cμ genes. 5 μg of Raji (**1**) or Raji-HN2 (**2**) DNA digested with BamHI, EcoRI, HindIII, PstI, SstI, or XbaI were electrophoresed in an agarose gel and blotted to Zetaprobe membrane. Separate blots were hybridized to nick-translated probes. The positions and sizes (*in* kb) of DNA molecular weight markers (λ/Hind III) are shown on the right. β-2m, β2-microglobulin.

Immunoglobulin Cμ

Raji is an early B lymphocyte characterized by the synthesis of the immunoglobulin IgM (Cμ sequences). Two size classes of Cμ mRNA have been reported: μm (about 2 kb) coding for membrane associated IgM; and μs (about 1.8 kb) coding for secreted IgM [26,27]. We probed a Northern blot of RNA from Raji and Raji-HN2 cells with a human Cμ probe and found that while similar amounts of Cμ mRNA were present in both cells, the mRNA found in Raji-HN2 cells was larger (Fig. 4). The μ mRNAs from Raji and Raji-HN2 cells were similar in size to μs and μm mRNA, respectively. Restriction enzyme analysis of the genomic Cμ sequences did not show any difference between the two cell lines (Fig. 5).

DISCUSSION

Burkitt lymphomas are aggressive lymphomas that respond to chemotherapy with alkylating agents [28]. A major limitation of chemotherapy is the acquisition by the treated tumor of resistance to the drug used in treatment. An understanding of the mechanism(s) of drug resistance is therefore important for developing strategies to

overcome or prevent the development of drug resistance. Because of the heterogeneity of cells present in primary tumors, tissue culture cell lines have been developed for studies of drug resistance. One such cell line, Raji-HN2, was developed to study the mechanism of resistance to alkylating agents [2]. Raji-HN2 cells are 10–20-fold more resistant than the parental Raji cells to the cytotoxic effects of HN2 but are not cross-resistant to other alkylating agents [2,29]. We reported the following changes in Raji-HN2 cells: a) increased extractable topoisomerase II activity, b) increased sensitivity to topoisomerase II inhibitors, c) increased sensitivity of chromatin to nuclease digestion, and, d) increased cell-doubling time [4,5]. Whether any of these changes plays a role in the mechanism of resistance to HN2, which is likely to be complex, is not known.

A major target of HN2 is cellular DNA. Because treatment of cells with DNA-reactive agents can cause gene alterations [21,22], we determined whether such alterations were induced in Raji-HN2 cells. To further our understanding of this cell system, we investigated the expression of three unrelated classes of genes, namely, housekeeping genes (actin and β2-microglobulin), a tumor specific gene (c-myc), and a cell-specific gene (immunoglobulin Cμ).

Of the four genes examined, only the mRNA of the actin gene were increased in Raji-HN2 cells; those of β2-microglobulin, c-myc oncogene, and immunoglobulin Cμ were present in equivalent amounts in both Raji and Raji-HN2 cells. The increased actin mRNA in Raji-HN2 cells may be the result of increased transcription or increased stability. Total actin protein and actin filaments were also detected in elevated amounts in Raji-HN2 cells. Because actins are highly conserved multigene family proteins [6,7], the probes that we have used do not allow us to identify which species of actin is increased in Raji-HN2 cells. The significance of the increased actin content of Raji-HN2 cells is not known; it may be required to maintain the shape of these enlarged cells.

Actin mRNA is frequently being used as an internal standard for quantitating RNA in blot analysis. Because we found that actin mRNA was increased in cells after an alteration in phenotype, the use of actin mRNA as an internal standard for the quantitative analysis of RNA may not be appropriate. An alternative method is to normalize the RNA under investigation against the poly(A) content of the sample by hybridization with labeled poly(T) [30].

Raji cell is an early B cell that synthesizes the heavy μ chain of the IgM class of immunoglobulin. The Cμ specific mRNA from Raji and Raji-HN2 cells corresponded in size to the mRNA for secreted and membrane-associated μ protein, respectively. Because antibody production is the only known function of B cells [12], the altered Cμ mRNA seen in Raji-HN2 cells is probably associated with an altered phenotype and unrelated to resistance to HN2.

Our studies show that the altered phenotype induced by HN2 is accompanied by the altered expression of certain genes. While the mechanism of resistance to HN2 remains to be elucidated, Raji and Raji-HN2 cells represent a valuable system for the study of gene regulation and its relationship to drug resistance.

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