Colchicine Quantification in Salt Stress Treated Culture of *Colchicum luteum* Baker by High Pressure Liquid Chromatography

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ABSTRACT

Objective: The aim of the present work was to develop a protocol for season independent propagation of *Colchicum luteum* and enrichment of colchicine yield under salt stress.

Materials and Methods: *C. luteum* was collected from Kashmir, India and was cultured in vitro. The callus was exposed to different NaCl (salt) treatments and the yield of colchicine was quantified by high pressure liquid chromatography (HPLC).

Results: Different explants viz. seeds, leaves, anthers and corm were used and the callus was only induced from corm segments. The callus induction and proliferation were best achieved on Murashige and Skoog medium supplemented with 2.0 mg L⁻¹ 2, 4-D + 4.0 mg L⁻¹ BAP. Direct and indirect plant regeneration from corm was observed in 2.0 mg L⁻¹ BAP + 2.0 mg L⁻¹ NAA added medium. The addition of adjuvants like activated charcoal and citric acid was noted to be less efficient for improving callus growth. Induced callus was elicited with different NaCl concentrations (T₀ = without NaCl, T₁ = 25, T₂ = 50, T₃ = 75, and T₄ =100 mM). The yield of colchicine was quantified by HPLC at periodic intervals (2, 4, and 6 weeks). All the used levels improved colchicine yield, but the content was maximum at T₃ (871.4 ng mg⁻¹ DWB). This is the first report of callus induction and plant regeneration from corm and the quantification of colchicine under salt stress in *C. luteum*.

Conclusion: Sodium chloride is thus a potential inducer and elicitor in improving colchicine yield in *C. luteum*.

Keywords: *Colchicum luteum*, exudates, regeneration, colchicine, in vitro cultures, stress

INTRODUCTION

*Colchicum luteum* Baker is rare to Jammu and Kashmir and belongs to the family Colchicaceae (1). It is commonly known as “Suranjan” or “Hiranatutiya” and in Kashmir the plant is known as “Vir keom”. The plant is popularly found on the edges of sub alpine forests and in open meadows in the temperate western Himalayas extending from Kashmir to Chamba at altitudes of between 700 and 2800 m. The plant bears conical-shaped corm with a middle groove, running longitudinally along its flat side, rapier (elongated and pointed) leaves, short scape and bright yellow flowers. The corms are wrapped with a dark brown thin cover, flattened on one side while the other side is rounded. Leaves are fewer in number and are approximately 15-30 cm in length and 0.8-1.5 cm broad. The alkaloid colchicine is extracted from *C. luteum* (2), and is used for...
the treatment of various diseases like Behçet’s syndrome (3) and gout - also known as “rich man’s disease”. Colchicum luteum possesses anti-cancerous activity due to colchicine and demecolcine (4). The corms of C. luteum are antinociceptive and anti-inflammatory (5).

Seed propagation of C. luteum is rather difficult because of its hard coat, low germination rate and long juvenile stage of about five years (6). Because of these difficulties in seed based propagation, young corms could be used as a good alternative source of different Colchicum species (7). Colchicine is used widely in plant improvement programmes and because of its other medicinal applications there arises an urgent need to conserve plant sources in vitro and ex situ. In vitro micro propagation provides a sensible technique for the conservation of rare and endangered plants (8).

The objective of the present work was to design a method for propagation of C. luteum and to enrich colchicine levels in cultivated tissues under NaCl elicitation/stress. To our best knowledge there is no report on callus culture from corm sections in C. luteum. The effect of salt stress in regulating the colchicine yield was also described.

MATERIALS AND METHODS

Collection of Plant Material

Colchicum luteum Baker plants (10 accessions each) were collected from different locations viz., Sonmarg, Gulmarg, Awantipora, Ferozpur, Aphanwat and Kralsangri of Kashmir province. The plants were very similar apart for one or two morphological attributes (corm, perianth). Plant material was authenticated and the herbarium of the same was submitted to the Centre for Biodiversity and Taxonomy, University of Kashmir, bearing voucher no. 2212-KASH.

Surface Sterilization of Explants

Different explants viz. leaves, corms, perianth, filaments, anthers and seeds were used during the present study. The explants were kept under running tap water for 30-45 min, the corms however, required more time (45-60 min). Later these explants were kept in labolene and tween for a further 15 min. The explants were sterilized with double distilled water to remove the traces of ethanol. Afterwards, these explants (other than corms) were surface sterilized in 4% sodium hypochlorite (10 min) while the corms were dipped in 0.1% mercuric chloride (HgCl₂) for 20 min and washed 3-4 times by using autoclaved water for 15 min to leach out alkaloids or other materials that would otherwise interfere with growth. After inoculation, the cultures were placed in an incubation room and were exposed to 16 h of photoperiodism. The MS medium used for the establishment of culture was supplemented with different PGRs. The different concentrations and combinations of PGRs were 0.5 mg L⁻¹ 2, 4-D, 0.5 mg L⁻¹ NAA, 0.5 mg L⁻¹ BAP, 0.5 mg L⁻¹ 2, 4-D + 0.5 mg L⁻¹ BAP, 1.0 mg L⁻¹ 2, 4-D + 1.0 mg L⁻¹ BAP, 0.5 mg L⁻¹ 2, 4-D + 0.5 mg L⁻¹ NAA and 1.0 mg L⁻¹ 2, 4-D + 1.0 mg L⁻¹ NAA. To initiate and proliferate callus at higher rate, other physico-chemical parameters tried were the effect of red light and the effect of GA₃ (0.25, 0.50 and 1.0 mg L⁻¹).

Scanning Electron Microscopy (SEM)

For SEM. callus with somatic embryos were collected from sub cultured medium. The tissue was fixed in 3% gluteraldehyde, washed with a 0.05 M potassium phosphate buffer (pH 7.0) and then dehydrated using ethanol. The sample was dried in a High Vacuum Evaporator (Hitachi-HUS 5GB). After gold plating, the sample was observed under a Scanning Electron Microscope (Hitachi S-3000).

Stress Treatment

Sodium chloride (NaCl) was added to the MS medium and dissolved properly prior to pH maintenance. The callus was then exposed with different concentrations of sodium chloride. The various concentrations used were T₁ = 25 mM, T₂ = 50 mM, T₃ = 75 mM and T₄ = 100 mM NaCl and a control (T₀ = 0 mM) was also kept for comparison.

HPLC

Extraction

The collected callus material was dried at room temperature for 1-2 weeks. The dried samples were ground using a pestle and mortar. Powder samples weighing 0.5 g were placed in a 50 ml conical flask containing 10 ml of Petroleum Ether, frequently shaken for 1 h, this process repeated, and finally the mixture was filtered using Watmann’s filter paper. The solid residues were dried in a vacuum, 10 ml of dichloromethane was added and then shaken regularly for 45 min. Then, 0.5 ml of 12.5% ammonium hydroxide was added followed by 15 min constant shaking. The cocktail was kept undisturbed for 30 min and the calli residues and the supernatant were preserved after filtration. About 10 ml of HPLC grade dichloromethane was used to wash the calli residues and was later filtered. The filtrates were collected and the organic phase was allowed to evaporate to dryness in a vacuum at room temperature and then dissolved in 10 ml of ethanol (70%). The mixture was filtered using filter paper and then transferred to 2.0 ml Eppendorf tubes. A few biochemical tests were carried out to confirm the presence of colchicine. An alcoholic solution of colchicine is red in colour when treated with ferric chloride (FeCl₃) and yellow in colour when treated with mineral acids.

Instrumentation

The analysis of the colchicine was done by using HPLC system called WATERS (Milford, USA) having Waters PDA 2998 series
photodiode array detector set at wavelength range 190-800 nm. The column from the Waters Spherisorb® C18 bonded with 5 µm (4.6 x 250 mm) accompanied with EMPOWER-2 software was utilized for collection and processing of chromatographic data. Ultrasonic cleaner (Steryl medi-equip systems) and water purification system ELIX 03 (MILLIPORE, USA) was also used.

**Sample Preparation**
Stock solution of colchicine (marker) of concentration 1 mg/ml was used. Other samples were dissolved in methanol and stored at a low temperature (4°C) until used. Different dilutions of stock solution with methanol were used to obtain 100 µg/ml so as to get standard curve.

**Optimization of Chromatographic Conditions**
Separation of compounds by HPLC is largely governed by the type of mobile phase used and organic modifiers. Different compositions, combinations, flow rate of organic solvents and other chromatographic parameters were used to standardize the protocol for optimization. The mobile phase combination of H2O: MeOH: Formic acid in the ratio of 50: 50:0.1 with isocratic elution at flow rate of 1.0 ml/min was used and found to be the optimum combination. For colchicine, best resolution and sensitivity was obtained at 352 nm. The chromatogram of sharp and symmetric peak of retention time 3.82 min was obtained under optimized conditions.

**Validation of Optimized Method**
After optimizing the chromatographic method, it was validated according to ICH guidelines for linearity, sensitivity, precision and recovery studies were carried out (ICH Guidelines, 1994; IC Guidelines, 1996).

**Calibration Curve (Linearity)**
The calibration curve linearity was drawn by using six different concentrations of colchicine (in triplicate) and the calibration curve was plotted in 1-100 µg/ml of colchicine range. The curve was plotted by replicate analysis at all concentration levels and the linear relationship was established using the least square method with Microsoft® Excel program.

**Table 1.** Callus induction (%) from corm in C. luteum, MS medium was amended with below mentioned PGRs.

| PGR (mg L⁻¹) | 2 weeks | 4 weeks | 6 weeks |
|-------------|---------|---------|---------|
| 0.25 2,4-D  | 15.0±2.88 a | 18.33±1.66 a | 21.66±4.40 b |
| 0.50 2,4-D  | 18.33±1.66 a | 20.00±5.00 b | 41.66±1.66 d |
| 1.0 2,4-D   | 35.00±2.88 c | 41.66±3.33 d | 43.33±1.66 d |
| 1.0 2,4-D + 2.0 BAP | 21.66±1.66 b | 23.33±1.66 b | 26.66±1.66 b |
| 2.0 2,4-D + 4.0 BAP | 53.33±3.33 e | 61.66±1.66 f | 76.66±1.66 e |
| 3.0 2,4-D + 6.0 BAP | 28.33±4.40 b | 31.66±1.66 c | 35.00±2.88 c |
| LSD at 5%   | 0.01     | 0.00    | 0.002   |

Values are means ± standard errors of 3 replicates. Within each column, values are followed by the same alphabet are not significantly different at p ≤ 0.05 level according to LSD.

**Precision**
Intra-day and inter-day precision for the developed method were calculated as percent relative standard deviation (% RSD). For intra-day precision, the experiments were repeated three times a day while for inter-day, three different days were considered. The concentration values for both intra-day and inter-day precision were calculated six times respectively and % RSD was similarly computed.

**Detection and Quantification Limits (LOD and LOQ)**
Limits of detection and quantification were calculated by method based on standard deviation (σ) and slope (S) of calibration plot using formula LOD = 3.3σ/S and LOQ = 10σ/S.

**RESULTS**
Different explants viz. corms, leaves, perianths, filaments, anthers and seeds of C. luteum plants from different geographical locations were tested in MS medium. Callus initiation was only achieved from corm explant (Figure 1a) irrespective of source regions. Seeds showed no response even after physical scarification (rubbing with sand paper) as the seeds possess a hard seed coat. The leaf and anther were also noted to be non responsive. Anthers turned black after a week of inoculation, perianth showed slight puffiness at the base of attachment after two weeks of inoculation and filaments showed bulging after two weeks of incubation. Anther, perianth and leaf all failed to produce callus in tested media conditions.

Different concentrations and combinations of PGRs were tried for callus production. In the first sets of experiments, 2, 4-D alone and in combination with BAP was used. From Table 1, it is apparent that the low auxin and high cytokinin concentrations / ratios were more efficient in inducing callus and for further growth (Figure 1b). Of all the combinations tested, 2.0 mg L⁻¹ 2, 4-D + 4.0 mg L⁻¹ BAP proved to have a good effect on callusing frequency. BAP alone had no effect on callus initiation. The induced callus initiated
from corm explant was yellowish, soft and exudated slimy viscous secretions. For a higher rate of callus initiation and proliferation, other methods using explant with GA₃ and exposing the explant in red light (by providing a red transparent cover to the vial) was noted ineffective. Callus biomass growth/proliferation was observed in different PGR combinations and concentrations but the rate of growth was highest (0.65 mg) in the 2.0 mg L⁻¹ 2, 4-D + 4.0 mg L⁻¹ BAP (Figure 2) added medium. The initial amount of callus placed in the MS medium was 0.15 mg and the biomass increase was monitored after 2, 4 and 6 weeks of culture. In order to adsorb phenolic exudates, various strategies like the addition of citric acid and activated charcoal were used as adjuvants. These adjuvants were added to the medium to see the influence of their effect on callusing and on growth by reducing callus browning, however, the response was noted to be non-promotive (Tables 2 and 3).

On sub culturing the callus on the same medium, the corm-callus transformed into embryogenic callus, which was distinctly different from non embryogenic tissue. Although the number of somatic embryos varied, on average 6-8 different stages of embryos (Figure 1c) were noticed per callus mass of about 200-300 mg. The number of embryos increased with each subculture. Moreover, the young corm-bud directly regenerated into shoots (Figure 1d) on MS medium. The embryo bearing callus was subjected to SEM studies to know more about the embryo details which revealed globular
embryos with stalk present on the surface of the callus (Figure 1e). The embryo developed on embryogenic callus on subculturing on shoot regeneration medium produced shoots (Table 4); the combination of 4.0 mg L$^{-1}$ BAP + 2.0 mg L$^{-1}$ NAA was noted to be the most efficient treatment for shoot development. For direct shoot regeneration, 2.0 mg L$^{-1}$ BAP + 2.0 mg L$^{-1}$ NAA supplemented medium was observed to be very effective (Table 5). All other treatments also showed some direct shoot regeneration ability with variable efficiency (33.33-40.66% after 6th weeks of culture).

The process of elicitation is an important technique to improve secondary metabolites in culture. Elicitors are molecules which activate plant defense mechanism and in turn improve alkaloid yield. In the present study, NaCl was used as an abiotic (salt) stress and the yield of colchicine was quantified in embryogenic

### Table 2. Effect of adjuvants on callus induction percentage, MS medium was supplemented with 2.0 mg L$^{-1}$ 2, 4-D + 4.0 mg L$^{-1}$ BAP

| Adjuvants               | Callus induction (%) |
|-------------------------|----------------------|
|                        | 2 weeks   | 4 weeks   | 6 weeks   |
| Control (without adjuvant) | 53.33±1.66$^c$ | 61.66±1.66$^c$ | 76.66±1.66$^c$ |
| Citric acid (10 g L$^{-1}$) | 41.66±1.66$^a$ | 45.00±2.88$^b$ | 48.33±4.40$^a$ |
| Activated charcoal (1.0 g L$^{-1}$) | 45.00±2.88$^b$ | 45.00±2.88$^b$ | 51.66±3.33$^b$ |
| LSD at 5%               | 0.021     | 0.005     | 0.011     |

Values are means ± standard errors of 3 replicates. Within each column, values are followed by the same alphabet are not significantly different at $p \leq 0.05$ level according to LSD.

### Table 3. Effect of adjuvants on callus biomass, MS was supplemented with 2.0 mg L$^{-1}$ 2, 4-D + 4.0 mg L$^{-1}$ BAP (initial weight = 0.15 mg)

| Adjuvants               | Callus proliferation (mg) |
|-------------------------|---------------------------|
|                        | 2 weeks   | 4 weeks   | 6 weeks   |
| Control (without adjuvants) | 0.20±0.02$^b$ | 0.30±0.02$^b$ | 0.58±0.01$^d$ |
| Citric acid             | 0.18±0.005$^a$ | 0.22±0.01$^b$ | 0.32±0.01$^c$ |
| Activated charcoal      | 0.19±0.005$^a$ | 0.21±0.008$^b$ | 0.23±0.006$^b$ |
| LSD at 5%               | 0.60       | 0.04       | 0.00       |

Values are means ± standard errors of 3 replicates. Within each column, values are followed by the same alphabet are not significantly different at $p \leq 0.05$ level according to LSD.

### Table 4. Effect of BAP and NAA at below concentrations on shoot development ability from embryogenic callus in C. luteum.

| PGR (mg L$^{-1}$) | Shoot development rate (%) |
|-------------------|-----------------------------|
|                   | 2 weeks   | 4 weeks   | 6 weeks   |
| 2.0 BAP+1.0 NAA   | 36.66±1.66$^a$ | 43.33±1.66$^b$ | 46.66±1.66$^b$ |
| 4.0 BAP+2.0 NAA   | 53.33±2.88$^c$ | 60.00±2.88$^d$ | 70.00±2.88$^d$ |
| 6.0 BAP+3.0 NAA   | 38.33±4.40$^a$ | 41.66±3.33$^b$ | 46.66±3.33$^b$ |
| LSD at 5%         | 0.01       | 0.006      | 0.001      |

Values are means ± standard errors of at least 3 replicates. Within each column, values are followed by the same alphabet are not significantly different at $p \leq 0.05$ level according to LSD.
cultures as these tissues were more differentiated and supposed to synthesize alkaloids much than the undifferentiated, non-embryogenic callus. The HPLC chromatograms of standard and treated culture are presented in Figure 3 and Figure 4. Almost all the concentrations of NaCl improved colchicine in tissues, but the content was at its maximum in T3 (133.6 ng mg⁻¹ DWB), followed by T4 (106.6 ng mg⁻¹ DWB) after 6th weeks of culture. As expected, the minimum level of colchicine was detected in T0 i.e. NaCl-free culture (Table 6). Results clearly indicate that the colchicine level improved with an increase in NaCl concentration, but at higher doses, the colchicine accumulation declined.

Table 5. Shoots directly regenerated from corm explants in C. luteum, MS medium was amended with below PGR combinations and concentrations.

| PGR (mg L⁻¹) | 2 weeks | 4 weeks | 6 weeks |
|--------------|---------|---------|---------|
| 1.0 2,4-D+1.0 BAP | 26.66±4.40b | 30.00±2.88b | 36.66±4.40b |
| 2.0 2,4-D+2.0 BAP | 23.33±3.33b | 30.00±2.88b | 35.00±2.88b |
| 3.0 2,4-D+3.0 BAP | 28.33±1.66b | 35.00±3.33b | 40.66±2.88b |
| 1.0 BAP+1.0 NAA | 25.00±2.88b | 28.33±1.66b | 33.33±3.33b |
| 2.0 BAP+2.0 NAA | 55.00±2.88d | 61.66±1.66a | 71.66±1.66d |
| 3.0 BAP+3.0 NAA | 31.66±1.66b | 36.66±1.66b | 40.00±2.88b |
| LSD at 5% | 0.00 | 0.00 | 0.01 |

Values are means ± standard errors of 3 replicates. Within each column, values are followed by the same alphabet are not significantly different at p ≤ 0.05 level according to LSD. (T₀ = without NaCl, T₁ = 25mM, T₂ = 50mM, T₃ = 75mM and T₄ = 100 mM NaCl).

Table 6. Effect of different concentrations of NaCl (salt) on colchicine content (ng mg⁻¹ DWB) in C. luteum after regular interval of time (2 weeks, 4 weeks and 6 weeks)

| NaCl | After 2 weeks | After 4 weeks | After 6 weeks |
|------|--------------|--------------|--------------|
| T₀   | 47.00±3.2b   | 55.00±6.0b   | 66.66±5.2c   |
| T₁   | 79.66±5.2c   | 85.33±4.0b   | 98.33±6.3c   |
| T₂   | 73.33±3.3b   | 87.33±6.1c   | 98.66±8.4b   |
| T₃   | 102.3±6.5c   | 112.0±5.6b   | 133.6±13.5f  |
| T₄   | 88.00±6.6c   | 101.0±7.9d   | 106.6±7.2c   |
| LSD at 5% | 0.00 | 0.001 | 0.004 |

Values are means ± standard errors of 3 replicates. Within each column, values are followed by the same alphabet are not significantly different at p ≤ 0.05 level according to LSD.
DISCUSSION

In this present investigation, the impact of NaCl was studied on colchicine yield in in vitro raised tissues in C. luteum. Seeds are the primary source of explants, but here in this present study, the seeds did not germinate, even after stratification. Mortezza et al. (10) reported similar response of no seed germination with stratification; hot temperature treatment, however, improved seed germination potentiality in C. kotschyi. As an alternative, other plant parts viz. leaves, corms, perianth, filaments and anthers were used for inducing callus and the corm was noted to be responsive only in producing callus. In another species of Colchicum (C. hierosolymitanum), callus was successfully induced from embryonic axis of seeds inoculated on 0.45 μM 2, 4-D supplemented medium (11). This explant-specific response is not new and has been noted in a large number of investigated cases (12). This differential callus forming ability may be due to the presence or absence of an endogenous level of PGRs and other physiological gradient in diverse explants (13). The callus was established in 2, 4-D added medium alone and in combination with BAP. The addition of PGRs particularly the auxins, was noted to be very promotive in producing callus in a number of studied plant materials (14,15). With a few exceptions of brown callus, the callus was mostly pale yellow, moderately hard, compact and slimy in nature. The browning may have been due to exudates present in corm which leached into the medium (11). This result is very similar to previous investigations on Origanum vulgare where phenolic browning occurred after two weeks of callus incubation (16). Shweta et al. (17) reported that the addition of citric acid (10.0 mg L⁻¹) in medium efficiently minimized the effect of phenolic exudation in C. luteum. In our present study, citric acid amendment had no significant effect on Colchicum cultures. The callus however, grew well and produced good amount of biomass (fresh weight) on increased concentration of BA (9 μM) and 2, 4-D (0.45 μM) in other investigated study and the observation (11) is similar and coincides with our present investigation.

The induced callus on sub culturing on the same 2,4-D containing medium transformed into embryogenic callus, distinctly different from non-embryogenic tissues on which a variable number of embryos were differentiated indirectly. No embryo was developed on the medium supplemented with NAA under light or dark conditions, a few embryos were, however, obtained on PGR-free medium. This indirect formation of embryo on callus was noted in several economically important plants (18). The influence of 2, 4-D/auxins on induction of callus and later on embryo formation was reported in several investigated cases (19,15). Nidal et al. (11) noted that the presence of 2, 4-D alone or in combination with BA was very effective in inducing callus and promoting somatic embryo numbers in C. hierosolymitanum culture. In this present study, we observed embryogenic callus on 2, 4-D and BAP added medium, confirmed by SEM studies. Scanning electron microscopic study has been widely used for the establishment and confirmation of somatic embryo mediated plant regeneration in a number of plant systems (20,21). The auxin’s molecular role in inducing callus/embryo is not known fully but several lines of research suggested that auxins upregulate genes under stress situations in acquiring embryogenic competence (22,23). Induced embryos matured and germinated into plants on a BAP added medium. The auxin germination/maturation is a key step in embryo-mediated propagation in which the addition of BAP or with NAA supplementation in medium facilitated plantlet production from embryos in other systems is used (24). The dormant corm bud was inoculated for shoot formation and within one or two weeks, outgrowths appeared as a first indicator of bud, which later turned into shoot primordia and grew moderately in the same BAP + NAA amended medium. Similar direct shoot primordial formation and their growth were noted in BAP, and with NAA added MS medium in many plants (25).

The present investigation was also conducted to see the impact of NaCl on colchicine yield in in vitro raised embryogenic tissues. The yield of colchicine was noted high in NaCl amended medium especially at T3. A similar enriched level of phyto-compounds following elicitation was reported in several investigated plant materials (26-28). The current study also revealed that the influence is dependent on NaCl concentration, the high to moderate level (75 mM) was more responsive in promoting colchicine to compare low NaCl levels (25 mM and 50 mM) and control. Earlier, in a similar approach Al-Fayyad et al. (29) quantified colchicine yield in corms (0.052%), flowers (0.025%) and leaves (0.013%) in Colchicum hierosolymitanum by using different NPK fertilizer levels. In this species (C. hierosolymitanum), the maximum yield of colchicine was also noted in callus added to 30 mM NH₄ and 0.1 M of sucrose (30). Sivakumar et al. (31) described the importance of biorhizomes, rhizome specific genes/gene which over expressed in rhizomes for the biomanufacture of colchicine as root, callus and cell suspension of G. superba and various species of Colchicum yielded poor level of colchicine. It is believed that the synthesis of colchicine and colchicoside, a related secondary metabolite are often controlled by various external factors; our results thus indicate that NaCl could be a good inducer in improving colchicine yield, therefore of huge practical applications. The synthesis of alkaloid has been noted to be more in differentiated embryogenic tissue and at the onset of somatic embryo formation (marked by the appearance of procambial tissue and rudiments of transport systems like vascular elements), the synthesis of alkaloids begins, this indicates that the yield content of alkaloid is directly related with embryogenesis (32). How and why synthesis is variable in different plant parts is still not known, some future experiments dealing with genes participating colchicine biosynthesis may help in unraveling the fact.

CONCLUSION

For callus induction, corn pieces proved to be the suitable explant, which produced callus on 0.5 mg L⁻¹ 2, 4-D and 0.5 mg L⁻¹ BAP added MS medium. In C. luteum, the combination of low auxin and high cytokinin concentration are necessary compared to the auxin alone. The growth of callus at the beginning is, however, slow. The induced callus transformed
into embryogenic tissue and later produced somatic embryos. The recent study suggests sodium chloride is a potential inducer in improving colchicine yield in *C. luteum*.

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**REFERENCES**

1. Kumar GP, Kumar R, Chaurasia OP, Singh SB. Current status and potential prospects of medicinal plant sector in trans-Himalayan Ladakh. J Med Plants Res 2011; 5: 2929-40.
2. Bashir A. Antioxidant activity and phenolic compounds from *Colchicum luteum* Baker (Liliaceae). Afr J Biotechnol 2010; 9: 5762-6.
3. Wechsler B. Colchicine and Behcet’s disease: An efficacious treatment finally recognized. J Med Int 2002; 23: 355-6.
4. Sakarkar DM, Deshmukh VN. Ethno pharmacological review of *Colchicum autumnale* L. Accessed on line at website: http://www.inchem. rg/documents/ ims/plant/ im142.htm. 2006; PP: 1-33.
5. Inchem. *Colchicum autumnale* L. Accessed on line at website: http://www.inchem. rg/documents/ims/plant/im142.htm. 2006; PP: 1-33.
6. Al-Fayyad M. Study of morphological characteristics, colchicine content and genetic variation of two wild *Colchicum* species in Jordan. Ph.D. Thesis, University of Jordan, Amman, Jordan. 2004.
7. Inc hem. *Colchicum autumnale* L. Accessed on line at website: http://www.inchem. rg/documents/ims/plant/im142.htm. 2006; PP: 1-33.
8. Naz R, Anis M. Acceleration of adventitious shoots by intersection between exogenous hormone and adenine sulphate in *Althaea officinalis* L. Appl Biochem Biotechnol 2012; 168:1239-55.
9. Murashige T, Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 1962; 15:473-97.
10. Morteza AN, Hossein A, Shamsali R, Mahmoud S, Yahya S, Navid M. Warm stratification and chemical treatments overcome the dormancy and promotes germination of *Colchicum kotschyi* Boiss seeds under *in vitro* condition. Not Sci Biol 2011; 3: 1004-1.
11. Nidal QO, Rida AS, Ibrahim MM, Feras A, Tamara SA. Callus Culture and Somatic Embryogenesis in *Wild Colchicum hierosolymitanum* Feib. Jordan J Agricultural Sciences 2012; 8: 356-356.
12. Ling APK, Tan KP, Hussein S. Comparative effects of plant growth regulators on leaf and stem explants of *Labisia pumila* var. alata. J Zhejiang Univ Sci B 2013; 14: 621-31.
13. Ghosh S, Sengupta S, Pal A. Abscissic acid, one of the key determinants of *in vitro* shoot differentiation from cotyledons of *Vigna radiate*. Amer J Pl Sci 2014; 5: 704 -13.
14. Su YH, Zhao XY, Liu YB, Zhang CL, O'Neill SD; Zhang X. Auxin-induced WUS expression is essential for embryonic stem cell renewal during somatic embryogenesis in *Arabidopsis*. Plant J 2009; 59: 448-60.
15. Fehéř A. Somatic embryogenesis - Stress-induced remodeling of plant cell fate. Biochem Biophys Acta 2015; 1849(4): 385-402.
16. Arefeh RM, Shibli RA, Al-Mahmoud M, Shatnawi MA. Callusing, cell suspension culture and secondary metabolites production in *Persian Oregano (Origanum vulgare L.)* and *Arabian Oregano (O. syriacum L.)*. Jordan J Agri Sci 2006; 2: 274-82.
17. Shweta S, Madhuparna B, Haider ZA. Conservation of *Colchicum luteum* Baker through micropropagation. J Research (BAU) 2013; 25: 96-100.
18. Zhao Y, Feng Y, Dai C. Effects of endophytic fungal elicitor on two kinds of terpenoids production and physiological indexes in *Euphorbia pekinensis* suspension cells. J Med Plants Res 2011; 5: 418-25.
19. Yang JL, Seong ES, Kim MJ, Ghimire BK, KANG WH, Yu CY, et al. Direct somatic embryogenesis from pericycle cells of *broccoli* (*Brassica oleracea* L. var. *italica*) root explants. Plant Cell Tiss Org Cult 2010; 100: 49-58.
20. Aslam M, Mujib A, Sharma MP. Somatic embryogenesis in *Catharanthus roseus*: a scanning electron microscopic study. Not Sci Biol 2015; 6 (2): 167-72.
21. Wang HC, Chen JT, Chang WC. Morphogenetic routes of long-term embryogenic callus culture of *Arceca catechu*. Biol Plant 2010; 54:1-5.
22. Gilwicka M, Nowak K, Balazadeh S, Mueller-Roeber B, Gaj MD. Extensive modulation of the transcription factor transcriptome during somatic embryogenesis in *Arabidopsis thaliana*. PloS ONE 2013; 8: e69261. doi: 10.1371/journal.pone.0069261.
23. Bai B, Su YH, Yuan J, Zhang XS. Induction of somatic embryos in *Arabidopsis* requires local YUCCA expression mediated by the down-regulation of ethylene biosynthesis. Mol Plant 2013; 6: 1247-60.
24. Mujib A, Ali M, Dipti T, Zafar N. Embryogenesis in ornamental monocots: Plant growth regulators as signaling element. In: Mujib, A., (ed). Somatic embryogenesis in ornamentals and its applications, Springer 2016 pp: 187-201.
25. Ashraf MF. Effect of cytokinin types, concentrations and their interactions on *in vitro* shoot regeneration of *Chlorophyllum borivilianum* Sant. & Fernandez. Electronic J Biotech 2014; 7: 275-9.
26. Ramakrishna A, Ravishankar GA. Influence of abiotic stress signals on secondary metabolites in plants. Plant Signal Behav 2011; 6: 1720-31.
27. Ribkahwati Purnobasuki H, Isnaeni Utami ESW. Quantity essential oil from rose callus leaf (*Rosa hybrid* L. variety Hybride tea purple): Results of light elicitation. J Chem Pharma Res 2015; 7: 496-9.
28. Samar F, Mujib A, Tonk D. NaCl amendment improves vinblastine and vincristine synthesis in *Catharanthus roseus*: a case of stress signalling as evidenced by antioxidant enzymes activities. Plant Cell Tissue Org Cult 2015; 121: 445-58.
29. Al-Fayyad M, Alali F, Alkofali A, Tell A. Determination of colchicine content and *Colchicum luteum* var. *alata* (*C. luteum*). Eur J Biol 2020; 79(2): 67-74.
30. Maqsood et al. Colchicine Quantification in Salt Treated *Colchicum luteum*.
Somatic Missense Mutations of Histone Variant H3.3 in Central Nervous System Cancers

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ABSTRACT

Objective: Histone variants are important modulators of chromatin functions. Studies have pointed out that epigenetic factors are often dysregulated in carcinogenesis. Although some cancer-associated mutations of the histone variant H3.3 have been identified previously, a complete list of H3.3 mutations and their potential effects is yet to be uncovered. Therefore, this study aims to identify the missense mutations of the histone variant H3.3 in central nervous system (CNS) cancers and to computationally predict their functional consequences on pathogenicity, protein stability and structure.

Materials and Methods: A complete set of human H3.3 mutations was acquired from the COSMIC v90 database and missense mutations were selected. The potential effects of these mutations were assessed using PredictSNP2 and FATHMM-XF. Structural outcomes were predicted using MUpro and HOPE servers.

Results: We identified 45 unique missense H3.3 substitutions in several tissues including CNS. PredictSNP2 and FATHMM-XF predicted 17 and 42 mutations as deleterious respectively, most of which caused decreased protein stability. Amino acid alterations in CNS cancers were predicted to cause alterations of the 3D structure.

Conclusion: Histone variants play significant roles in epigenetic regulation and are often mutated in cancers. Our results showed that H3.3 mutations detected in CNS cancers could affect the genomic distribution of post-translational modifications and histone variants, hence dramatically alter the gene expression profile and contribute to carcinogenesis.

Keywords: Epigenetics, histone variant H3.3, mutation analysis, cancer

INTRODUCTION

Epigenetic regulation is a phenomenon that modulates cellular processes such as proliferation, progression through the cell cycle, transcriptional memory, and DNA damage repair via regulating accessibility of DNA through chromatin condensation (1). The genetic material of eukaryotic organisms exists within the cell as a complex macromolecule called chromatin, consisting of DNA and histone proteins. The packaging of DNA into chromatin dictates differential gene expression patterns which are crucial for the proper functioning of the cell. Perturbations in these processes, as well as transcriptional regulation mechanisms are often associated with complex diseases such as cancer. These regulatory mechanisms are orchestrated by DNA methylation, RNA interference, post-translational histone modifications and incorporation of histone variants into chromatin. Histones are small, basic proteins encoded by several copies of histone genes located within the major histone locus. Canonical histones, namely H2A, H2B, H3 and H4 are strictly synthesized during the S-phase and deposited onto the chromatin in a replication-dependent manner. Histone variants, however, are expressed throughout the cell cycle and incorporated into chromatin in a context-dependent manner (2). Canonical histones and their corresponding variants differ in their amino acid sequence, which affects interactions between histone proteins within the same nucleosome and results in alterations in transcriptional activity. The most commonly studied histone variants
are centromere specific H3 variant CENP-A (3); DNA damage site specific H2AX (4); H2AZ and H3.3 variants that are commonly found at active transcription sites (5); and macroH2A which is associated with transcriptional repression (6).

Tumors of the brain and the spinal cord, which collectively comprise the central nervous system (CNS), are amongst the most heterogeneous cancer types. The World Health Organization classifies CNS cancers into more than 120 subtypes based on molecular and histopathological characteristics (7). CNS tumors often originate from different cell types, such as astrocytes, glia and meninges. Gliomas and meningiomas are the major subtypes of brain tumors in adults and are rarely seen (8). On the contrary, CNS cancers are the most common solid tumor type in children between the ages of 0-14 (9), 30% of which is constituted by medulloblastomas (10). Gliomas are graded at four levels depending on the severity, aggressiveness and curability of the disease. Medulloblastoma is the common name for a group of malignant embryonic tumor subtypes that originate from the primitive neuronal cells within the posterior cranial fossa (11). Although the etiology of CNS cancers remains unknown to date, several genetic factors have been associated with increased risk. For instance, a recent study indicated the significant contribution of germline mutations and a genetic disposition to pediatric medulloblastoma (12). Similarly, the mutational status of TP53, BRAF, FGFR1, IDH and TERT as well as the copy number variations of EGFR, CDKN2A/B, PTEN, PDGFRA are often linked with tumor pathogenesis in the CNS (13,14). These genetic variations also serve as powerful molecular tools for cancer subtype characterization.

Studies have shown that several epigenetic factors, including histone variants, are mutated or their activities are dysregulated during cancer pathogenesis. Although previous studies have identified some cancer-associated mutations of the histone variant H3.3 in chondroblasia, pediatric sarcoma, giant cell tumor of bone, glioma and medulloblastoma (15-17), a complete list of H3.3 mutations and their potential effects is yet to be uncovered. Therefore, this paper aims to identify H3.3 mutations in CNS cancers and to predict their functional consequences on pathogenicity, protein stability and structure using computational approaches.

MATERIALS AND METHODS

Identification of Deleterious Mutations

The functional consequences and the pathogenicity scores of the H3.3 mutations were predicted using PredictSNP2 (https://loschmidt.chemi.muni.cz/predictsnp2/) (18) and FATHMM-XF (http://fathmm.biocompute.org.uk/fathmm-xf/index.html) (19) in reference to genome assembly GRCh38/hg38.

Protein Stability Prediction

The effect of the missense mutations on the stability of the protein was analyzed via MUpro using H3.3 amino acid sequence retrieved from UniProt (ID: P84243), which is based on machine learning methods (http://mupro.proteomics.ics.uic.edu/) (20). The tool provides 84.2% accuracy.

Determination of 3D Structural Changes

The structural effects of the nonsynonymous H3.3 mutations, which were commonly encountered in CNS cancers were predicted via HOPE server using H3.3 amino acid sequence retrieved from UniProt (ID: P84243) (https://www3.cmbi.umcn.nl/hope) (21).

In silico Evaluation of H3.3 Conservation

The amino acid sequences of histone H3.3 for Homo sapiens (P84243-1), Mus musculus (P84244-1), Rattus norvegicus (P84245-1), Gallus gallus (P84247-1), Xenopus laevis (Q6PI79-1), Danio rerio (Q6PI20-1), Saccharomyces cerevisiae (P10651-1) and Arabidopsis thaliana (P59169-1) were retrieved from UniProt. In silico evaluation of protein similarity was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (22). Histone domain structure was determined in accordance with Luger et al. (23).

RESULTS

Identification of H3.3 Mutations

COSMIC database analysis over 1012 unique samples showed that 94.2% of all somatic H3.3 mutations were missense substitutions (n=953), most of which were caused by A>T nucleotide change. Furthermore, 2 nonsense substitutions, 8 synonymous substitutions, 2 frameshift deletions, 1 inframe insertion, 1 inframe deletion and 2 uncharacterized mutations were detected. The missense mutations were detected in various tissue types including but not limited to breast, cervix, prostate, upper aerodigestive tract, bone and central nervous system tissues. Table 1 shows a complete list of 45 nonsynonymous mutations identified in H3.3. Proteins often undergo N-terminal methionine cleavage by methionine aminopeptidase (MAP), which removes the first methionine coded by the start codon (24). Therefore, the starting methionine is not always present in the mature protein. We realized that the locations of the amino acid substitutions identified by the COSMIC database analysis differ from the mature protein by one amino acid, since the COSMIC database did not take the methionine removal into account. The amino acid changes before (detected by COSMIC) and after MAP cleavage are shown in Table 1. For coherence with other studies in the literature, our further analyses were based on the locations of amino acid substitutions after N-terminal methionine cleavage.

Prediction of Pathogenicity and Protein Stability

The potential effects of somatic H3.3 mutations on carcinogenesis were predicted using two methods. PredictSNP2 calculates an expected accuracy value, which is the consensus classifier for prediction of the effects of nucleotide variants based on 5 different nucleotide-based prediction tools (CADD, DANN, FATHMM, FunSeq2 and GWAVA). PredictSNP2 analysis revealed that 17
Table 1. Computational predictions of pathogenicity and protein stability for the H3.3 mutations.

| CDS mutation | AA mutation (COSMIC) | AA mutation (MAP cleavage) | PredictSNP2 Analysis | FATHMM-XF Analysis | MUpro Analysis |
|--------------|----------------------|---------------------------|----------------------|-------------------|---------------|
|              |                      |                           | Pathogenicity prediction | Expected accuracy | Pathogenicity score | Stability prediction | Confidence score |
| c.7C>T       | p.R3C                | p.R2C                      | Neutral               | 65%               | 0.580          | Decrease           | -0.639           |
| c.14A>T      | p.K5M                | p.K4M                      | Neutral               | 63%               | 0.860          | Increase           | 0.133            |
| c.17A>C      | p.Q6P                | p.Q5P                      | Neutral               | 63%               | 0.941          | Decrease           | -0.469           |
| c.18G>C      | p.Q6H                | p.Q5H                      | Neutral               | 67%               | 0.436          | Decrease           | -0.481           |
| c.25C>G      | p.R9G                | p.R8G                      | Neutral               | 63%               | 0.913          | Decrease           | -1.865           |
| c.25C>T      | p.R9C                | p.R8C                      | Neutral               | 63%               | 0.879          | Decrease           | -0.853           |
| c.26G>A      | p.R9H                | p.R8H                      | Neutral               | 63%               | 0.873          | Decrease           | -1.566           |
| c.34A>G      | p.T12A               | p.T11A                     | Neutral               | 65%               | 0.839          | Decrease           | -1.544           |
| c.37G>A      | p.G13S               | p.G12S                     | Neutral               | 63%               | 0.882          | Decrease           | -1.347           |
| c.60A>C      | p.Q20H               | p.Q19H                     | Neutral               | 89%               | 0.323          | Decrease           | -0.804           |
| c.67A>C      | p.T23P               | p.T22P                     | Deleterious           | 82%               | 0.940          | Decrease           | -1.642           |
| c.76G>T      | p.A26S               | p.A25S                     | Neutral               | 65%               | 0.878          | Decrease           | -1.333           |
| c.82A>G      | p.K28E               | p.K27E                     | Neutral               | 63%               | 0.874          | Decrease           | -0.218           |
| c.83A>T      | p.K28M               | p.K27M                     | Deleterious           | 82%               | 0.874          | Decrease           | -1.566           |
| c.84G>T      | p.K28N               | p.K27N                     | Neutral               | 63%               | 0.559          | Decrease           | -0.414           |
| c.86G>C      | p.S27T               | p.S28T                     | Neutral               | 63%               | 0.863          | Decrease           | -0.496           |
| c.98C>T      | p.T33I               | p.T32I                     | Neutral               | 63%               | 0.856          | Decrease           | -0.256           |
| c.103G>T     | p.G35W               | p.G34W                     | Deleterious           | 82%               | 0.920          | Decrease           | -0.756           |
| c.103G>A/C   | p.G35R               | p.G34R                     | Deleterious           | 87%               | 0.912          | Decrease           | -0.739           |
| c.104G>T     | p.G35V               | p.G34V                     | Deleterious           | 87%               | 0.917          | Decrease           | -0.746           |
| c.110A>T     | p.K37M               | p.K36M                     | Neutral               | 63%               | 0.851          | Increase           | 0.315            |
| c.111G>T     | p.K37N               | p.K36N                     | Neutral               | 65%               | 0.687          | Decrease           | -0.176           |
| c.118C>A     | p.H40N               | p.H39N                     | Deleterious           | 87%               | 0.890          | Increase           | 0.148            |
| c.136A>G     | p.T46A               | p.T45A                     | Neutral               | 63%               | 0.855          | Decrease           | -0.494           |
| c.139G>A     | p.V47M               | p.V46M                     | Neutral               | 63%               | 0.888          | Decrease           | -0.013           |
| c.143C>A     | p.A48E               | p.A47E                     | Neutral               | 63%               | 0.889          | Decrease           | -0.334           |
| c.148C>T     | p.R50C               | p.R49C                     | Deleterious           | 87%               | 0.647          | Decrease           | -0.127           |
| c.149G>A     | p.R50H               | p.R49H                     | Neutral               | 65%               | 0.892          | Decrease           | -0.768           |
| c.160C>T     | p.R54C               | p.R53C                     | Deleterious           | 82%               | 0.910          | Decrease           | -1.181           |
| c.168G>T     | p.Q56H               | p.Q55H                     | Deleterious           | 82%               | 0.577          | Decrease           | -1.236           |
| c.190C>T     | p.R64C               | p.R63C                     | Neutral               | 67%               | 0.909          | Decrease           | -1.283           |
| c.218G>A     | p.R73Q               | p.R72Q                     | Deleterious           | 82%               | 0.869          | Decrease           | -0.505           |
| c.244G>A     | p.D82N               | p.D81N                     | Deleterious           | 87%               | 0.878          | Decrease           | -0.620           |
| c.245A>T     | p.D82V               | p.D81V                     | Deleterious           | 87%               | 0.881          | Decrease           | -0.105           |
| c.262G>T     | p.A88S               | p.A87S                     | Deleterious           | 82%               | 0.636          | Decrease           | -0.748           |
| c.268A>G     | p.J90V               | p.J89V                     | Neutral               | 65%               | 0.480          | Decrease           | -0.920           |
| c.295G>A     | p.A99T               | p.A98T                     | Neutral               | 67%               | 0.836          | Decrease           | -1.258           |
| c.299A>G     | p.Y100C              | p.Y99C                     | Neutral               | 63%               | 0.906          | Decrease           | -1.230           |
| c.317A>T     | p.E106V              | p.E105V                    | Neutral               | 63%               | 0.918          | Decrease           | -0.412           |
| c.344C>G     | p.A115G              | p.A114G                    | Deleterious           | 82%               | 0.924          | Decrease           | -1.305           |
| c.371A>G     | p.D124G              | p.D123G                    | Deleterious           | 82%               | 0.913          | Decrease           | -1.351           |
| c.378G>T     | p.Q126H              | p.Q125H                    | Deleterious           | 87%               | 0.792          | Decrease           | -1.024           |
| c.385S>T     | p.R129C              | p.R128C                    | Neutral               | 65%               | 0.926          | Decrease           | -0.823           |
| c.386G>A     | p.R129H              | p.R128H                    | Neutral               | 63%               | 0.912          | Decrease           | -1.120           |
| c.389G>A     | p.R130H              | p.R129H                    | Deleterious           | 82%               | 0.915          | Decrease           | -1.542           |
out of 45 nucleotide substitutions were deleterious mutations with more than 82% expected accuracy. Higher percentage values indicate higher confidence; hence, G34R/V, H39N, R49C and D81N/V substitutions had the most confident pathogenicity prediction percentages (87%).

A second analysis was performed using FATHMM-XF, which produces p-values (pathogenicity scores) between 0-1 and predicts mutations with p>0.5 as pathogenic/deleterious. FATHMM-XF analysis yielded 42 mutations predicted to be deleterious.

Lastly, the effect of H3.3 mutations on the overall protein stability was predicted using the MUpro tool, which calculates a confidence score, the prediction of the value of energy change (delta delta G), using a machine learning approach, namely the Support Vector Machine. Values <0 indicate decreased protein stability, while values >0 suggest increased protein stability, showing that most of the mutations caused decreased protein stability with varying confidence scores. Amino acid substitutions with the lowest confidence scores, which are R8G, R8H, T11A, K27M and R129H, are predicted to result in a greater decrease in protein stability.

**H3.3 Mutations in CNS**

Among the analyzed set of unique H3.3 missense mutations, the majority were identified in CNS (77%) and bone (14%) (Figure 1). Therefore, we selected the ones that originated from the CNS as the primary site for further analysis. H3.3 mutations in the CNS namely p.R2C, p.R8H, p.K27M, p.G34R, p.G34R, p.G34W and p.G34V, were mainly derived from brain, cerebral hemisphere, temporal lobe and frontal lobe, while they were also detected to a

| AA mutation | CDS mutation | Primary tissue | Tissue subtype | Histology |
|-------------|--------------|---------------|----------------|-----------|
| p.R2C       | c.7C>T       | CNS           | Brain          | Glioma, primitive neuroectodermal tumour-medullo-blastoma |
| p.R8H       | c.26G>A      | CNS           | Brain          | Glioma, primitive neuroectodermal tumour-medullo-blastoma |
| p.K27M      | c.83A>T      | CNS           | Brain, cerebral hemisphere, thalamus, temporal lobe, spinal cord, posterior fossa, brainstem | Glioma, primitive neuroectodermal tumour-medullo-blastoma |
| p.G34R      | c.103G>A     | CNS           | Brain, cerebral hemisphere, occipital lobe, frontal lobe, parietal lobe, temporal lobe | Glioma, primitive neuroectodermal tumour-medullo-blastoma |
| p.G34R      | c.103G>C     | CNS           | Basal ganglia, cerebral hemisphere, temporal lobe | Glioma, primitive neuroectodermal tumour-medullo-blastoma |
| p.G34W      | c.103G>T     | CNS           | Frontal lobe   | Glioma |
| p.G34V      | c.104G>T     | CNS           | Brain, frontal lobe | Glioma |
lesser degree in thalamus, spinal cord, posterior fossa, brain stem, occipital lobe, parietal lobe, temporal lobe and basal ganglia (Table 2). Histologically, the tissues exhibited properties of glioma and primitive neuroectodermal tumour-medulloblastoma.

Analysis of Structural Alterations

Size, charge, and hydrophobicity are significant features of amino acids which critically differentiate them from one another. Therefore, newly introduced mutant residues often result in structural alterations within the protein. We assessed these potential 3D alterations using the HOPE server (Figure 2). For p.R2C and p.R8H substitutions, the side chains of the two amino acids were significantly different. The mutant residue was smaller and had a different charge when compared to the wild-type protein. p.K27M substitution resulted in a smaller mutant residue, while it was more hydrophobic than the wild-type residue. Substitution of G34 to R, V and W caused bigger and more hydrophobic mutant residues. p.G34R and p.G34W gave rise to the incorpo-

![Figure 2. The 3D constructions depicting structural changes due to missense substitutions R2C, R8H, K27M, G34R, G34V and G34W that are found in glioma. Wild-type and mutated amino acids are indicated in green and red respectively.](image)

![Figure 3. Alignment of histone H3.3 amino acid sequences in human, mouse, rat, chicken, frog, zebra fish, yeast and plant tissues using Clustal Omega. N- and C-terminal tails and histone fold regions (α1-2-3 helices and L1-2 loops) are indicated. Amino acids R2, R8, K27 and G34 (marked by green) are conserved across species.](image)
ration of significantly different side chains into the protein. The amino acid sequence alignment of histone H3.3 from human, mouse, rat, chicken, frog, zebrafish, yeast and plant tissues indicated significant conservation across species (Figure 3). All mutant residues given in Figure 2 were located within the highly conserved N-terminal tail of histone H3.3, which is an important domain for interaction with other molecules.

DISCUSSION

Histone H3 has three main variants; while H3.1 and H3.2 are classified as the canonical histone H3, H3.3 is the so-called "replacement variant" which gets synthesized and incorporated into the chromatin throughout the cell cycle (25). Although the amino acid sequence of H3.3 differs only slightly from the canonical H3, most of these variant residues lie within structurally important domains that affect its interaction with other histones in the nucleosome and histone chaperones (26). Therefore, nucleosomes containing H3.3 usually confer an open chromatin conformation and are found at active or poised transcription sites that are enriched in tri-methylations of histone H3 at lysine 4 and 27 (27,28). Genes encoding epigenetic factors such as histones, their modifiers and chaperones, as well as chromatin remodeling enzymes are often mutated in cancer and are linked with tumorigenesis (29,30). Studies that have previously identified H3.3 mutations in various cancers mostly focused on the amino acid substitutions at K27, G34 and K36. Mutations in K27 were mainly found in diffuse intrinsic pontine glioma (DIPG), a subtype of pediatric glioma, and were associated with poor prognosis (15-17). G34 substitutions were found in pediatric gliomas located in cerebral hemispheres and giant cell tumors of the bones in young adults (15,31,32). K36 mutations were identified in chondroblastomas and pediatric sarcomas (17,33). In this paper, we identified the whole set of H3.3 mutations in addition those previously identified and characterized (17,33). Among all the somatic missense mutations of histone variant H3.3 retrieved from the COSMIC database across different tissues, the majority were found in the central nervous system cancers. These mutations can potentially act through two mechanisms: either by affecting histone PTMs or altering interactions between histones and their chaperones. Most of these mutation hotspots are functionally critical as they can be post-translationally modified, which is a crucial mechanism for epigenetic regulation of transcriptional activity. H3K27me3 is a repressive histone modification located in transcriptionally inactive genes and compact chromatin loci (34,35). Substitution of lysine at position 27 to methionine abolishes this function and results in decreased H3K27me3 levels since methionine cannot be methylated. In line with this, it was previously reported that patients with p.K27M substitutions exhibit globally reduced H3K27me3 levels (36). H3K36me3 is found in actively transcribed gene bodies and missense mutations resulting in p.K36M/N cause reduced H3K36me3 levels (26). Glycine is not directly modified, but it is an amino acid that provides flexibility to the protein, which could be required for its proper functioning; thus, its mutation could result in disrupting this function. Furthermore, due to its proximity to K36, it is suggested that G34 substitutions could affect the function of H3K36me3 (32). In addition to these previously identified mutations, histone H3 can be methylated at arginine 2 and 8 by PRMT6 and PRMT5, respectively (37,38). Both of these post-transcriptional modifications are considered as repressive marks (37-40). Substitutions of arginines at positions 2 and 8 would prevent these methylations to take place and alter the transcriptional profile. Therefore, p.R2C and p.R8H could be as important as K27M and G34R/V/W in CNS tumorigenesis, although they are observed less frequently.

We also showed that all H3.3 mutations detected in CNS cancers introduced structural changes into the protein in varying degrees, which might be affecting its interactions with other proteins, such as histone chaperons. Histones are composed of N- and/or C-terminal tails and three α-helices connected by two loops, which is called the "histone fold motif" (41). This structure is highly conserved and is significant for interacting with both chromatin modulators and the DNA itself. For instance, histone tails that protrude from the nucleosome often interact with the DNA and contribute to the higher-order chromatin formation (42). Furthermore, histone variant H3.3 is deposited onto chromatin by two main histone chaperon complexes, namely HIRA and DAXX/ATRX (43); and disruption of these interactions would drastically change both the genomic distribution of histone variants and the transcriptional activity. Interestingly, patients with H3.3 mutations are reported to have frequent co-occurring mutations in DAXX and ATRX (44, 45). Almost all patients with mutated H3.3 and DAXX/ATRX also exhibit activation of a mechanism called "alternate lengthening of telomeres", which is a hallmark of cancer (44).

CONCLUSION

Incorporation of histone variant H3.3 into chromatin, as well as its somatic missense mutations dramatically alter the epigenomic landscape and the gene expression profile of a cell. Previous studies suggested that distinct gene expression patterns (45), transcriptome and interactome profiles (46) are established between tumors carrying different H3.3 mutations. Therefore, H3.3 mutations in CNS cancers are considered both as disruptors of the expression of genes required for brain function and as drivers of tumorigenesis (44,46). In conclusion, histone variants play significant roles in epigenetic regulation. Therefore, mutations in histone variant genes often contribute to carcinogenesis. A better understanding of these cancer-related mutations and their potential effects is useful for future studies.

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REFERENCES

1. Kouzarides T. Chromatin modifications and their function. Cell 2007; 6(4): 693-705.
2. Albig W, Doenecke D. The human histone gene cluster at the D6S105 locus. Hum Genet 1997; 101: 284-94.
3. Yoda K, Ando S, Morishita S, Houmura K, Hashimoto K, Takeyasu K, et al. Human centromere protein A (CENP-A) can replace histone H3 in nucleosome reconstitution in vitro. Proc Natl Acad Sci USA 2000; 97: 7266-71.
4. Rogakou EP, Boon C, Redon C, Bonner WM. Megabase chromatin domains involved in DNA double-strand breaks in vivo. J Cell Biol 1999; 146: 905-16.
5. Thakar A, Gupta P, Ishibashi T, Finn R, Silva-Moreno B, Uchiyama S, et al. H2A.Z and H3.3 histone variants affect nucleosome structure: biochemical and biophysical studies. Biochemistry 2009; 48(46): 10852-7.
6. Chakravarthy S, Gundimella SK, Caron C, Perche PY, Pherson JR, Knochbin S, et al. Structural characterization of the histone variant macroH2A. Mol Cell Biol 2005; 25: 7616-24.
7. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization classification of tumors of the central nervous system: a summary. Acta Neuropathol 2016; 131(6): 803-20. doi: 10.1007/s00401-016-1545-1.
8. McNell KA, Epidemiology of brain tumors. Neuroul Clin 2016; 34(4): 981-98. http://dx.doi.org/10.1016/j.ncl.2016.06.014.
9. Ostrom QT, de Blank PM, Kruchko C, Petersen CM, Liao P, Finlay JL, et al. The glioblastoma position in medulloblastoma: a retrospective genetic study and prospective validation in a clinical trial cohort. Lancet Oncol 2018; 19(5): e246-e56. doi: 10.1016/s1470-2045(18)30242-0.
10. Park SH, Won J, Kim SI, Lee Y, Park CK, Kim SK, et al. Molecular testing of brain tumor. J Pathol Transl Med 2017; 51(3): 205-23. doi:10.4132/jptm.2017.03.08.
11. Aldape K, Zadeh G, Mansouri S, Reifenberger G, von Deimling A. Glioblastoma: pathology, molecular mechanisms and markers. Acta Neuropathol 2015; 129: 829-48.
12. Khung-Quang DA, Buczkwowicz P, Rakopoulos P, Liu XY, Fontebasso AM, Bouffet E et al. K27M mutation in histone H3.3 defines clinically and bioactively distinct subgroups of pediatric diffuse intrinsic pontine gliomas. Acta Neuropathol 2012; 124(3): 439-47. doi:10.1007/s00401-012-0998-0.
13. Wan YCE, Liu J, Chan KM. Histone H3 mutations in cancer. Curr PharmacoRep 2018; 4(4): 292-300. doi: 10.1007/s40495-018-0141-6.
35. Black JC, Van Rechem C, Whetstone JR. Histone lysine methylation dynamics: establishment, regulation, and biological impact. Mol Cell 2012; 48(4): 491-507. doi: 10.1016/j.molcel.2012.11.006.

36. Bender S, Tang Y, Lindroth AM, Hovestadt V, Jones DT, Kool M, et al. Reduced H3K27me3 and DNA hypomethylation are major drivers of gene expression in K27M mutant pediatric high-grade gliomas. Cancer Cell 2013; 24(5): 660-72. doi: 10.1016/j.ccr.2013.10.006.

37. Guccione E, Bassi C, Casadio F, Martinato F, Cesaroni M, Schuchlautz H, et al. Methylation of histone H3R2 by PRMT6 and H3K4 by an MLL complex are mutually exclusive. Nature 2007; 449: 933-7.

38. Pal S, Vishwanath SN, Erdjument-Bromage H, Tempst P, Sif S. Human SWI/SNF-associated PRMT5 methylates histone H3 arginine 8 and negatively regulates expression of ST7 and NM23 tumor suppressor genes. Mol Cell Biol 2004; 24: 9630-45.

39. Hyllus D, Stein C, Schnabel K, Schiltz E, Imhof A, Dou Y, et al. PRMT6-mediated methylation of R2 in histone H3 antagonizes H3 K4 trimethylation. Genes Dev 2007; 21: 3369-80.

40. Wang L, Pal S, Sif S. Protein arginine methyltransferase 5 suppresses the transcription of the RB family of tumor suppressors in leukemia and lymphoma cells. Mol Cell Biol 2008; 28: 6262-77.

41. Arents G, Moudrianakis EN. The histone fold: a ubiquitous architectural motif utilized in DNA compaction and protein dimerization. Proc Natl Acad Sci USA 1995; 92(24): 11170-4. doi: 10.1073/pnas.92.24.11170.

42. Iwasaki S, Iwasaki W, Takahashi M, Sakamoto A, Watanabe C, Shichino Y, et al. The translation inhibitor rocaglamide targets a bimolecular cavity between eIF4A and polypurine RNA. Mol Cell 2019; 73(4): 738-748.e9. doi: 10.1016/j.molcel.2018.11.026.

43. Goldberg AD, Banaszynski LA, Noh KM, Lewis PW, Elsaesser SJ, Stadler S, et al. Distinct factors control histone variant H3.3 localization at specific genomic regions. Cell 2010; 140(5): 678-91. doi: 10.1016/j.cell.2010.01.003.

44. Yuen BT, Knoepfler PS. Histone H3.3 mutations: a variant path to cancer. Cancer Cell 2013; 24(5): 567-74. doi: 10.1016/j.ccr.2013.09.015.

45. Schwartzentruber J, Korshunov A, Liu XY, Jones DT, Pfaff E, Jacob K, et al. Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma. Nature 2012; 482(7384): 226-31. doi: 10.1038/nature10833.

46. Lim J, Park JH, Baude A, Fellenberg J, Zustin J, Haller F, et al. Transcriptome and protein interaction profiling in cancer cells with mutations in histone H3.3. Sci Data 2018; 5: 180283. doi: 10.1038/sdata.2018.283.